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**CEREBROSPINAL FLUID MARKERS  
FOR THE EARLY AND DIFFERENTIAL  
DIAGNOSIS OF  
ALZHEIMER'S DISEASE**

**Niki Schoonenboom**

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**VRIJE UNIVERSITEIT**

**Cerebrospinal fluid markers  
for the early and differential  
diagnosis of  
Alzheimer's disease**

**ACADEMISCH PROEFSCHRIFT**

ter verkrijging van de graad Doctor aan  
de Vrije Universiteit Amsterdam,  
op gezag van de rector magnificus  
prof.dr. L.M. Bouter,  
in het openbaar te verdedigen  
ten overstaan van de promotiecommissie  
van de faculteit der Geneeskunde  
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door

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geboren te Woerden

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## LIST OF ABBREVIATIONS

|                |   |
|----------------|---|
| A $\beta$      | Amyloid $\beta$   |
| A $\beta$ 1-42 | Full length A $\beta$ 42 peptide  |
| A $\beta$ N-42 | Full length A $\beta$ 42 and A $\beta$ peptides truncated at the N-terminus |
| A $\beta$ 38   | Amyloid $\beta$ 38  |
| A $\beta$ 40   | Amyloid $\beta$ 40  |
| A $\beta$ 42   | Amyloid $\beta$ 42  |
| $\alpha$ 1-ACT | $\alpha$ 1-antichymotrypsin   |
| AD             | Alzheimer's disease   |
| Apo E          | Apolipoprotein E  |
| APP            | Amyloid precursor protein   |
| AUC            | Area under the curve  |
| CDR            | Clinical dementia rating scale  |
| CI             | Confidence interval   |
| CJD            | Creutzfeldt-Jakob disease   |
| CSF            | Cerebrospinal fluid   |
| DLB            | Diffuse lewy body disease   |
| EAD            | Early-onset Alzheimer's disease   |
| EC             | Entorhinal cortex   |
| ELISA          | Enzyme-linked immunosorbent assay   |
| FTLD           | Frontotemporal lobar degeneration   |
| FTD            | Frontotemporal dementia   |
| IL             | Interleukin   |
| IP-10          | Interferon- $\gamma$ -inducible protein-10                                  |
| LAD            | Late-onset Alzheimer's disease  |
| LP             | Lumbar puncture   |
| LR             | Likelihood ratio  |
| MCI            | Mild Cognitive Impairment   |
| MCP-1          | Monocyte chemotactic protein-1  |
| MMSE           | Mini-mental state examination   |



## LIST OF ABBREVIATIONS

|                  |   |
|------------------|---|
| MRI              | Magnetic resonance imaging  |
| MTA              | Medial temporal lobe atrophy  |
| NFT              | Neurofibrillary tangles   |
| NINCDS-<br>ADRDA | National Institute of Neurological and Communicative Disorders and Stroke-<br>the Alzheimer's disease and Related Disorders Association |
| NP               | Neuritic plaques  |
| OR               | Odds ratio  |
| PAS              | Preclinical Alzheimer's disease scale   |
| PET              | Positron Emission Tomography  |
| PIB              | Pittsburgh Compound-B   |
| PSEN             | Presenilin  |
| Ptau             | Phosphorylated tau  |
| Ptau-181         | Tau phosphorylated at threonine 181   |
| Ptau-231         | Tau phosphorylated at threonine 231   |
| Ptau-199         | Tau phosphorylated at serine 199  |
| ROC              | Receiver operating curves   |
| SD               | Standard deviation  |
| SP               | Senile plaques  |
| Tau              | Total tau protein   |
| TMT              | Trailmaking test  |
| VAD              | Vascular Dementia   |
| VAT              | Visual association test   |

## **CHAPTER 1**

### **GENERAL INTRODUCTION**



## 1.1 Alzheimer's disease, mild cognitive impairment and neuropathological changes

The diagnosis 'possible and probable' AD is based on clinical criteria (see Appendix for description of the NINCDS-ADRDA criteria<sup>1</sup>) supported by neuropsychological tests, neuroimaging and follow up. A person is demented when he suffers from dysfunction in at least two cognitive domains as well as in daily activities, under the condition that the patient has a clear conscience. AD is defined as 'early onset' AD (EAD), when the symptoms start before the age of 65 years.<sup>2</sup> This definition is arbitrary, and based on historic decisions regarding the pension age only. Alois Alzheimer in 1907 described the first AD patient, being a 51-year old woman, and considered AD as a presenile type of dementia, while there existed already another poorly defined senile type of dementia in elderly persons. Almost sixty years later, it was found that this senile type of dementia exhibited the same neuropathological findings as in presenile AD.<sup>3</sup> The arbitrary distinction between the two types of AD still exists, but more and more evidence is gathered that distinguishes subtypes of AD based on other findings than age.<sup>4,5,83</sup> Still, EAD is considered to be a rare condition, although the frequency of EAD in a presenile dementia population is rather high.<sup>6</sup> Some autosomal dominant cases are associated with mutations in the amyloid precursor protein (APP) and presenilin (PSEN) genes.<sup>7</sup> However, in the majority of sporadic EAD no mutation was found.<sup>8</sup> The presence of one or more  $\epsilon 4$ -alleles of the Apolipoprotein E (Apo E) genotype is a risk factor for sporadic AD and is associated with an earlier age-at-onset.<sup>84-86</sup> Yet, Apo E gene polymorphism is not used in the diagnostic process of AD, as it has low specificity and sensitivity and little predictive value in an individual patient.<sup>88</sup> A definite diagnosis AD can only be obtained at autopsy, which holds true for most other types of dementia too. The clinicopathological correlation differs between centers and is around 70-80% overall.<sup>9</sup> On microscopic examination AD is characterized by a combination of abnormalities: diffuse and neuritic plaques (NP), containing extracellular non-fibrillar amyloid  $\beta$  ( $A\beta$ ), predominantly  $A\beta_{42}$ , and fibrillar  $A\beta_{42}$  respectively; intraneuronal neurofibrillary tangles (NFT) composed of abnormally phosphorylated tau (Ptau); and loss of synaptic proteins and neurons.<sup>10</sup>  $A\beta_{42}$  is formed by proteolytic processing of APP (see also Appendix). The pattern of distribution of NP and NFT throughout the brain differs with development of disease; in early preclinical stages NFT are found in the entorhinal cortex (EC) and hippocampus, and with progression of disease they spread via the medial temporal neocortex to other cortical areas. On the other hand,

NP deposition starts in the neocortical frontal/temporal areas and they spread in later stages to the hippocampal areas. The staging system developed by Braak and Braak describe the extent of tangle related abnormalities in AD, which correlates well with severity of dementia<sup>11</sup>. NP correlate less with cognitive dysfunction in AD patients.<sup>12</sup>

Plaques and, to a lesser extent, tangles are also found in brains of elderly non-demented controls and patients with mild cognitive impairment (MCI).<sup>13,14</sup> MCI is a clinical entity<sup>15</sup>, describing patients with subjective and/or objective cognitive complaints and mild functional disabilities, but no dementia. A variety of definitions exist for the concept MCI in literature. In our studies we included patients with ‘amnesic MCI’, conceptualized according to the criteria of Petersen et al.<sup>16</sup> According to these criteria patients have MCI when they have subjective and objective problems with short term memory compared to persons with the same age, with no interference in daily activities thus no dementia (see also Appendix). These amnesic MCI patients have a high chance of developing AD in the future, especially when they are over 70 years of age.<sup>15</sup> A few autopsy studies investigated whether NFT and NP are related to memory function in MCI patients<sup>13,14</sup>, NFT in the medial temporal lobe seem to have a relation with memory function in amnesic MCI. However, all these studies must be interpreted with caution as there might be a selection bias. The results might depend on the definition used for the concept MCI. In the next chapter (**1.2**) an overview is presented of studies describing the most promising biochemical markers in cerebrospinal fluid (CSF) for the (early) diagnosis AD which are supposed to reflect the neuropathological changes in AD. **Chapter 2** describes the aim of the present thesis in detail.

## **CHAPTER 1.2**

### **Cerebrospinal fluid markers for the diagnosis of Alzheimer's disease**

Niki Schoonenboom, Harald Hampel, Philip Scheltens, and Mony de Leon

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## Introduction

Alzheimer's disease (AD) is considered to be the most common type of dementia.<sup>1</sup> Due to the aging of the population, the number of persons affected by AD is expected to increase 3-fold by 2050.<sup>2</sup> The diagnosis AD is made by exclusion and based on clinical criteria<sup>3</sup>, supported by neuropsychological tests, neuroimaging and extended follow-up. In the early stage, it is difficult to differentiate AD from other types of dementia, as the clinical symptoms are subtle and the diagnostic methods may be normal. Furthermore, clinical overlap exists between the different types of dementias, while volume changes of the hippocampus and medial temporal lobe on magnetic resonance imaging (MRI) are not specific for AD.<sup>4</sup> With the advent of novel therapeutic strategies<sup>5</sup>, it became important to diagnose AD as early as possible, as pharmacological treatment needs to be started before extensive and irreversible brain damage has occurred. Over the last decade, many studies have been set out to find an appropriate biomarker for the diagnosis of AD.<sup>6</sup> This chapter starts with an overview as regards the most promising cerebrospinal fluid (CSF) biomarkers for the early and differential diagnosis of AD. Next, the relation of the biomarkers with atrophy on MRI will be discussed. Finally, limitations and topics for future research will be presented.

## Neuropathology

The basis for the research on biochemical markers are the neuropathological changes present in the various types of dementias.<sup>7</sup> Neuropathological hallmarks of AD -accumulation of extracellularly senile plaques (SP) and neurofibrillary tangles (NFT), synaptic reductions and neuron loss- gradually accumulate in time, and start long before the clinical picture of AD becomes overt.<sup>8</sup> SP are divided into two types: diffuse and neuritic plaques (NP). NP are composed of the highly insoluble fibrillar protein amyloid  $\beta$  42 ( $A\beta$ 42).  $A\beta$  depositions tend to accumulate with age. NFT are intraneuronal accumulations of abnormally (hyper)phosphorylated tau protein. NFT can be found already in non-demented subjects in the hippocampus and EC, the regions affected earliest in AD. NP initially are found in the neocortex, but in later stages they also affect the EC and the hippocampus.<sup>9,10</sup> Patients with frontotemporal dementia (FTD) show heterogeneity in underlying pathology<sup>11</sup>, with tau deposits in some of them. Creutzfeldt Jakob disease (CJD) is characterized by spongiform changes, neuronal loss, gliosis and immunostaining of the protease-resistant prion protein.<sup>12</sup> Dementia with Lewy Bodies (DLB) is part of the  $\alpha$ -



synucleinopathies, in which  $\alpha$ -synuclein accumulates in the intraneuronal Lewy Bodies.<sup>13</sup> Vascular dementia (VAD) is characterized by ischemic lesions, lacunes and extensive white matter changes.<sup>14</sup> Between the different types of clinically diagnosed dementias significant neuropathological overlap exists.<sup>15</sup> Lewy bodies are present in AD, whereas in DLB, FTD and VAD plaques and tangles can be found. White matter changes are found in all types of dementia, especially in AD.<sup>16</sup>

### **CSF amyloid $\beta$ 42 and tau in AD versus controls**

According to criteria established in 1998, a good biomarker has to have a sensitivity of at least 85% for AD and a specificity of  $\geq 75\%$  to differentiate AD from other types of dementia.<sup>7</sup> The most promising CSF markers to differentiate AD from non-demented elderly are A $\beta$ 42 and tau. Below, each biomarker will be discussed separately. Next, the most valid studies will be summarized for the combination of CSF A $\beta$ 42 and tau.

#### *A $\beta$ 42*

In numerous studies it has been shown that A $\beta$ 42 is decreased in CSF of AD patients compared to non-demented controls.<sup>6,17</sup> The decrease of A $\beta$ 42 concentration in CSF is thought to be the result of several mechanisms:

1. Deposition of insoluble A $\beta$ 42 in the SP of the brain, which might be in part the result of disturbance of the clearance of A $\beta$ 42
2. Decrease of production of A $\beta$ 42 by less (active) neurons, inevitably a result of neurodegeneration
3. Altered binding to A $\beta$ 42-specific proteins (e.g. Apo E), resulting in masking of the epitope, to which the antibodies of the assays are directed.

The concentration of CSF A $\beta$ 42 in AD is about 50% of that recorded in controls.<sup>17</sup> The most commonly used assay is the commercial ELISA of Innogenetics (Table 1). The median values of A $\beta$ 42, as measured in two large case-control studies, are:

AD: 487 (394-622) pg/mL, controls: 849 (682-1063) pg/mL.<sup>18</sup>

AD: 394 (326-504) pg/mL, controls: 1076 (941-1231) pg/mL.<sup>19</sup>

Reference value for CSF A $\beta$ 42 obtained from a control population is set above 500 pg/mL.<sup>20</sup> Sensitivity ranged from 69-100%, while specificity ranged from 56-85% in a subset of studies.<sup>19,21-25</sup> Considerable variability in absolute levels of A $\beta$ 42 exists among centers, even when using the same commercial assay. Cross-sectional studies show little evidence of a relationship between CSF A $\beta$ 42 and age, except for one study showing a U-shaped natural course in normal aging, with an increase of CSF A $\beta$ 42 until 29 and over 60 years old.<sup>26</sup> No<sup>18,23</sup> or only a weak<sup>22</sup> cross-sectional relation has been found between CSF A $\beta$ 42 and disease duration or mini-mental state examination (MMSE). Only one study investigated and found an association between the number of SP and the CSF A $\beta$ 42 concentration.<sup>27</sup>

### *Tau*

Many studies have demonstrated that tau is increased in CSF of AD patients; concentrations are about three times higher in AD than in non-demented controls. However, there is a large variation in the range of CSF tau concentration in AD. Median and mean concentrations of CSF are:

AD: 425 (274-713) pg/mL and 587 (365) pg/mL.<sup>6,18</sup>

Controls: 195 (121-294) pg/mL and 224 (156) pg/mL.

The increase of tau in CSF is supposed to be the result of release from dying neurons containing a large number of NFT. One study demonstrated that CSF tau concentration was related to the number of NFT in the brain.<sup>28</sup>

Again, the most common used assay for tau is the ELISA from Innogenetics (Table1). Mean sensitivity ranged from 55 to 81% at a mean specificity value of 90% comparing AD with controls<sup>17</sup>. Important is that CSF tau increases with age<sup>19,29</sup>, which stresses the need to compare only groups from the same age category.<sup>30</sup> Furthermore, CSF tau tends to be increased in several other neurological disorders, such as acute stroke<sup>31</sup>, and trauma<sup>32</sup>, indicating that the marker is not very specific. Reference values for tau in healthy individuals are defined as:

< 300 pg/mL (21-50 years)

< 450 pg/mL (51-70 years)

< 500 pg/mL (71-93 years).<sup>19</sup>

No correlation was found between CSF tau and MMSE or disease duration.

### *Combination of CSF A $\beta$ 42 and tau*

Diagnostic accuracy, especially the specificity, increases when using the combination of CSF A $\beta$ 42 and tau comparing AD with controls, including patients with depression or memory problems due to alcohol abuse.<sup>17</sup> In Table 1 an overview is given of class IA and 1A case-control studies, with neuropathological (IA) or clinical diagnosis (1A) as gold standard, and patient and control groups included with a minimum of thirty individuals.<sup>33</sup>

### *Isoprostanes*

Oxidative stress is thought to play an important role in the cascade resulting in cell death in AD.<sup>36</sup> A few studies have demonstrated that isoprostanes are increased in CSF of AD patients, even already at an early stage of disease.<sup>37,38</sup> Further studies are needed how these proteins can be used in the diagnostic work up for AD, especially to clarify the specificity of these markers.

## **CSF markers in AD versus other dementias**

### *Combination of CSF A $\beta$ 42 and tau*

How good is the diagnostic accuracy when using the combination of A $\beta$ 42 and tau in AD compared to other types of dementias? Although this topic is much more relevant for clinical practice, only a few studies investigated these two markers in large groups of patients. Most studies found a lower specificity as compared to the studies mentioned in Table 1. There is substantial overlap in CSF A $\beta$ 42 and tau concentrations between different types of dementias. A decreased concentration of CSF A $\beta$ 42 can be found in DLB, FTD, and VAD.<sup>18,20,30,39</sup> A high CSF tau is also not specific for AD: CSF tau is found to be increased in a subset of FTD and VAD patients.<sup>18,30</sup> In most cases of DLB CSF tau concentration is normal.<sup>39</sup> In CJD, CSF A $\beta$ 42 is decreased and CSF tau is found to be very high, even higher than in AD.<sup>40</sup> The specificity of the combined CSF A $\beta$ 42/tau analysis varies from 85% comparing AD with FTD to 67% in AD versus DLB and 48% in AD versus VAD (Table 2).

### *Phosphorylated tau*

Several investigators have developed assays to detect phosphorylated tau (Ptau) in CSF. As NFT are abundant of abnormally phosphorylated tau, it is to be expected that Ptau is increased in CSF from AD patients. Several immunoassays have been developed that are specific for the

**Table 1 Diagnostic accuracy of CSF A $\beta$ 42 and tau combined in AD versus controls**

| Study                               | Population  | Gold standard  | Criteria               | Result                                 | Cut off                                     | Method   |
|-------------------------------------|---|--|------------------------|--|---|--|
| Galasko, 1998 <sup>22</sup>         | 82 probable AD<br>60 controls                               | Clinical diagnosis (1A)                                  | NINCDS-ADRDA           | Sensitivity 77%<br>Specificity 93%     | A $\beta$ 42: 1032 pg/mL<br>Tau: 503 pg/mL  | A $\beta$ 42 and tau: In house methods<br>Multi-center study |
| Kanai, 1998 <sup>34</sup>           | 93 probable AD<br>41 controls                               | Clinical diagnosis (1A)                                  | NINCDS-ADRDA           | Sensitivity 40%<br>Specificity 90%     | A $\beta$ 42: 256 fmol/mL<br>Tau: 474 pg/mL | A $\beta$ 42: In house method<br>Tau: Innogenetics           |
| Hulstaert, 1999 <sup>18</sup>       | 150 probable AD<br>100 controls = 42 HC + 58<br>OND         | Clinical diagnosis (1A)                                  | NINCDS-ADRDA           | Sensitivity 85%<br>Specificity 86%     | A $\beta$ 42: 643 pg/mL<br>Tau: 252 pg/mL   | A $\beta$ 42 and tau: Innogenetics<br>Multi-center study     |
| Tapiola, 2000 <sup>23</sup>         | 80 probable AD<br>41 definite AD<br>39 OND                  | Clinical (1A) and<br>neuropathological<br>diagnosis (IA) | NINCDS-ADRDA<br>CERAD  | Sensitivity 46-53%*<br>Specificity 95% | A $\beta$ 42 : 340 pg/mL<br>Tau : 380 pg/mL | A $\beta$ 42: In house method<br>Tau: Innogenetics           |
| Andreasen, 2001 <sup>35</sup>       | 105 probable AD<br>100 controls of Hulstaert<br>et al. 1999 | Clinical diagnosis (1A)                                  | NINCDS-ADRDA           | Sensitivity 94%<br>Specificity 89%     | A $\beta$ 42: 643 pg/mL<br>Tau: 252 pg/mL   | A $\beta$ 42 and tau: Innogenetics                           |
| Riemenschneider, 2002 <sup>20</sup> | 74 probable AD<br>40 controls                               | Clinical diagnosis (1A)                                  | NINCDS-ADRDA           | Sensitivity 92%<br>Specificity 95%     | A $\beta$ 42: 738 pg/mL<br>Tau: 255 pg/mL   | A $\beta$ 42 and tau:<br>Innogenetics                        |
| Kapaki, 2003 <sup>24</sup>          | 49 probable AD<br>49 controls                               | Clinical diagnosis (1A)<br>3 year follow-up              | NINCDS-ADRDA           | Sensitivity 96%<br>Specificity 86%     | A $\beta$ 42: 490 pg/mL<br>Tau : 317 pg/mL  | A $\beta$ 42 and tau: Innogenetics                           |
| Sunderland, 2003 <sup>6</sup>       | 131 probable AD<br>72 controls                              | Clinical diagnosis (1A)                                  | DSM-IV<br>NINCDS-ADRDA | Sensitivity 92%<br>Specificity 89%     | A $\beta$ 42 : 444 pg/mL<br>Tau: 195 pg/mL  | A $\beta$ 42: In house method<br>Tau: Innogenetics           |

\* Definite and probable AD vs OND. Probable AD = AD according to the clinical NINCDS-ADRDA criteria; definite AD = AD confirmed at neuropathological examination; OND = other neurological diseases. 1A = clinical diagnosis is gold standard, prospective collected materials, including groups of patients and controls with a minimum of 30 individuals; IA neuropathological diagnosis is gold standard, rest conform class 1A.

**Table 2 Diagnostic accuracy of CSF A $\beta$ 42 and tau combined in AD versus other types of dementia**

| Study                               | Population                                 | Gold standard  | Criteria   | Result  | Cut off   |
|-------------------------------------|--|--|--|---|---|
| Galasko, 1998 <sup>22</sup>         | 82 probable AD<br>74 NAD                   | Clinical diagnosis (1A)                                  | NINCDS-ADRDA                                     | Sensitivity 77%<br>Specificity 65%                            | A $\beta$ 42: 1032 pg/mL<br>Tau: 503 pg/mL                |
| Hulstaert, 1999 <sup>18</sup>       | 150 probable AD<br>79 NAD                  | Clinical diagnosis (1A)                                  | NINCDS-ADRDA                                     | Sensitivity 85%<br>Specificity 58%                            | A $\beta$ 42: 643 pg/mL<br>Tau: 252 pg/mL                 |
| Tapiola, 2000 <sup>23</sup>         | 80 probable AD<br>41 definite AD<br>27 NAD | Clinical (1A) and<br>neuropathological<br>diagnosis (IA) | NINCDS-ADRDA<br>CERAD                            | Sensitivity 50%<br>Specificity 85%                            | A $\beta$ 42 : 340 pg/mL<br>Tau : 380 pg/mL               |
| Andreasen, 2001 <sup>35</sup>       | 105 probable AD<br>23 VAD<br>9 DLB         | Clinical diagnosis (1A)                                  | NINCDS-ADRDA<br>VAD: NINDS-AIREN<br>DLB: McKeith | Sensitivity 94%<br>Specificity VAD 48%<br>Specificity DLB 67% | A $\beta$ 42: 643 pg/mL<br>Tau: 252 pg/mL                 |
| Riemenschneider, 2002 <sup>20</sup> | 74 probable AD<br>34 FTLD                  | Clinical diagnosis (1A)                                  | NINCDS-ADRDA<br>FTLD: Neary                      | Sensitivity 85%<br>Specificity 85%                            | A $\beta$ 42: 528 pg/mL<br>Tau: 432 pg/mL                 |
| Kapaki, 2003 <sup>24</sup>          | 49 probable AD<br>15 NAD<br>6 VAD          | Clinical diagnosis (1A)                                  | NINCDS-ADRDA<br>VAD: NINDS-AIREN                 | AD vs NAD/VAD:<br>Sensitivity: 71-90%<br>Specificity: 83-100% | AD vs NAD:<br>A $\beta$ 42 : 435 pg/ml<br>Tau : 437 pg/ml |
| Schoonenboom, 2004 <sup>30</sup>    | 47 probable EAD<br>28 FTLD                 | Clinical diagnosis (1A)                                  | NINCDS-ADRDA<br>FTLD: Neary                      | Sensitivity 72%<br>Specificity 89%                            | A $\beta$ 42 : 413 pg/mL<br>Tau: 377 pg/mL                |

Probable AD = AD according to the clinical NINCDS-ADRDA criteria; definite AD = AD confirmed at neuropathological examination; EAD = early onset AD, disease starting before 65 years old;

VAD = vascular dementia; DLB = diffuse lewy body disease; FTLD = frontotemporal lobar degeneration; NAD = non-Alzheimer-dementia. 1A = clinical diagnosis is gold standard, prospective collected materials, including groups of patients and controls with a minimum of 30 individuals; IA neuropathological diagnosis is gold standard, rest conform class 1A.

phosphorylated epitopes threonine 181 (Ptau-181)<sup>41</sup>, serine 199 (Ptau-199)<sup>42</sup>, and threonine 231 (Ptau-231).<sup>43</sup> Good results have been obtained comparing AD with other types of dementia; in the majority of patients Ptau is found to be normal in DLB<sup>44</sup>, VAD<sup>45</sup>, FTD<sup>30</sup>, and CJD.<sup>46</sup> One study demonstrated an increase in diagnostic accuracy of Ptau-231 and Ptau-181 compared to Ptau-199 in differentiating AD from other types of dementia.<sup>47</sup> The same authors found a decline of CSF Ptau-231 during the course of AD in 17 patients.<sup>48</sup> These data need to be confirmed in another independent study, preferably with post-mortem confirmation of diagnoses. A greater diagnostic accuracy of Ptau compared to total tau is obtained in most studies.<sup>30,49</sup> In one study it has been shown that the combination of CSF A $\beta$ 42 with Ptau-181 differentiated best (early onset) AD (EAD) from FTD with a high specificity (93%) and a low negative predictive value (negative likelihood ratio: 0.03).<sup>30</sup> As there still exists overlap between the different types of dementia, either clinically or biochemically, a combination of the three markers seems best for routine clinical practice, with at least two of the three biomarkers positive as indicator for AD.<sup>50</sup>

#### *14-3-3 protein*

The 14-3-3 protein gives, like tau, a reflection of (fast progressive) neuron loss. It can be detected in CSF by the semi-quantitative method Western Blot analysis. When used in the proper context, with a high clinical suspicion and in combination with EEG, MRI scan and routine CSF analysis, the measurement of 14-3-3 protein in CSF supports the diagnose CJD with high diagnostic accuracy.<sup>51</sup> False-positive results can be obtained in acute stroke, brain tumor, encephalitis, or even (fast progressive) AD. Sensitivity and specificity values of CSF 14-3-3 and tau have been reported to be the same in one study (cut off level for tau = 1300 pg/mL).<sup>52</sup> Recently it has been shown that the combination of 14-3-3 protein and A $\beta$ 42 gives the highest diagnostic accuracy for CJD (sensitivity 100%, specificity 98%, positive predictive value 93%, negative predictive value 100%).<sup>40</sup>

#### **Gold standard**

The majority of above-mentioned studies have been obtained in groups of patients where the diagnosis has been obtained clinically. Accuracy of the clinical diagnosis in specialized settings is estimated around 85%.<sup>53</sup> By use of clinical criteria there is risk of circular reasoning -ie, the diagnostic performance of CSF markers cannot be higher than the accuracy of the clinical criteria.<sup>17</sup> The NINCDS/ADRDA criteria for AD have a high sensitivity but a moderately high specificity. Illustrative is the specificity of only 23% of the

NINCDS/ADRDA criteria for the differentiation of AD from FTD in one retrospective neuropathological study.<sup>54</sup> Furthermore, 40-80% of the clinically diagnosed VAD patients have concomitant AD pathology.<sup>55</sup> Only two studies were published in which (in part) the neuropathological diagnosis was used as gold standard.<sup>23,56</sup> For the differentiation of AD from controls similar sensitivity and specificity was obtained for CSF tau and A $\beta$ 42 as compared to clinical studies (Table 1<sup>23</sup>). However, specificity of FTD and DLB as compared to AD was not optimal, 69%.<sup>56</sup>

Most published studies were done in specialized tertiary referral settings with selected patient groups. Only a few studies were done with consecutively recruited patients from a memory clinic; sensitivity was high, but specificity was lower in this setting with 'unselected' patients.<sup>35,57</sup> More studies are needed in large primary and secondary referral centers to get insight how to use CSF A $\beta$ 42, tau and Ptau in an elderly population in clinical practice. Population-based studies are under way to establish CSF markers as potential biomarkers for routine diagnostic use.

### **Mild cognitive impairment**

Mild cognitive impairment (MCI) is considered to be a transitional state between normal aging and dementia. Around 10-15% of the MCI patients progress to Alzheimer type dementia each year.<sup>58</sup> Several studies have shown that a subgroup of MCI patients has low CSF A $\beta$ 42 levels and/or high CSF tau levels at baseline, that are indicative for AD.<sup>17</sup> Furthermore, there is evidence that these markers can be used as predictors for the conversion of MCI to AD.<sup>50,59</sup> It is not clear yet which marker is changing first in the disease process, as contradictory findings are reported by various studies describing either an increased CSF tau<sup>50,60</sup> or a decreased CSF A $\beta$ 42 at baseline.<sup>61,62</sup> In two independent studies a relation between CSF tau with memory impairment was found, while this was not the case for CSF A $\beta$ 42.<sup>63,64</sup> Good results have been obtained for CSF Ptau as indicator of AD-related changes in the MCI stage.<sup>4,59,65</sup> In one study it has been demonstrated that high CSF levels of Ptau at baseline, but not CSF tau levels, correlated with cognitive decline and conversion of MCI to AD.<sup>66</sup> A very recent study, following 78 MCI patients, shows the best prediction for the development of AD using the combination of CSF A $\beta$ 42 with Ptau.<sup>67</sup> Most of the studies mentioned have been conducted retrospectively in research settings, and limited data are available about the frequency of a biomarker profile typical for AD in a prospective setting that reflects clinical practice. But overall, the use of biomarkers in combination with other

diagnostic tools is very promising in recognizing MCI patients who will develop AD in the future.

## **Neuroimaging and CSF biomarker studies**

### *Cross-sectional studies*

Hippocampal size reduction, atrophy of the medial temporal lobe (MTL) and the entorhinal cortex are sensitive markers for AD. Moreover, atrophy of the hippocampus is found to be a good predictor in MCI for the development of AD. However, these markers are not disease-specific and cannot be used as primary evidence for AD.<sup>4</sup> By combining CSF and MRI markers one could get a better diagnostic accuracy. In addition, by investigating the relation between the two markers a better understanding of the agreement between the two disease markers could be obtained: do they reflect the same pathological substrate at the same time? Only a few studies investigated the cross-sectional relation between CSF biomarkers and atrophy on MRI in small groups of patients. One study showed a correlation between CSF A $\beta$ 42 and the volume of the temporal lobes.<sup>68</sup> We were unable to find a relationship between medial temporal lobe atrophy (MTA), and CSF A $\beta$ 42, tau and Ptau in 62 mild-moderate AD patients and 32 controls when considered as separate groups.<sup>69</sup> Moreover, both disease markers contributed independently to the diagnose AD. In MCI patients, we found a relation between CSF A $\beta$ 42 and MTA, while CSF tau did not relate to MTA.<sup>63</sup> These data corresponded to a larger study reporting lower baseline CSF A $\beta$ 42 levels with lower brain volume and larger ventricular volume in the spectrum of normal aging, MCI and AD.<sup>70</sup> In contrast, higher CSF tau and Ptau were found with an increase in ventricular widening during follow up. In this light, CSF A $\beta$ 42 can be more considered as a *stage* marker, indicating the presence of disease at a certain time, while CSF tau is more a *state* marker, indicating the intensity of the neuronal damage and degeneration.<sup>17,70</sup> However, these data give only information about one time-point in disease, and until yet it was not possible to show progressive changes in CSF A $\beta$ 42 or (P)-tau concentrations, except for one study.<sup>71</sup> On the other hand, atrophy rates on MRI are good indicators of disease progression in MCI and AD. The question is therefore: can one or both disease markers be used as markers of progression?



### *Longitudinal studies*

The few studies investigating the change in CSF biomarkers were done in AD patients. Little is known about the change of CSF A $\beta$ 42, tau and Ptau in MCI, while one would expect that in this early stage of disease the biomarkers are more prone to change than in later stages. One study investigated whether there was a longitudinal relationship between the change in biomarkers with the change in hippocampal volume on MRI in a small group of aged individuals with and without memory problems.<sup>4</sup> In a 2 time-point longitudinal design, the MCI group, N=8, showed an inverse relationship between hippocampal volume reductions and elevations in CSF Ptau, while CSF A $\beta$ 42 levels showed a positive relation with hippocampal volume reductions. However, there are several limitations of this study: a very small group was investigated; it is not known whether these MCI patients will develop AD; and the change in biomarkers could also be due to the intra-assay variability, as very small changes are detected. The authors did not find a significant change in CSF A $\beta$ 42, tau and Ptau between two time points, only if they corrected for dilution of Ptau due to ventricular enlargement; this 'Ptau-231 load' was increased in MCI at follow-up.<sup>65</sup> These findings need to be replicated in larger groups of patients, while additional studies are warranted for a better understanding of CSF flow and clearance dynamics of biomarkers.

### **Additive value of CSF markers over other diagnostic tools**

In a recent review the position of CSF markers in the clinical assessment of patients with MCI and early AD has been discussed.<sup>17</sup> The authors suggest that only after intensive screening of the patients by history, neurological examination, routine laboratory tests (blood and CSF), and neuroimaging (CT, MRI or SPECT) there is place for CSF markers for the (early) diagnosis of AD. The clinical diagnosis of AD should be based on cumulative information of all the different diagnostic tools, as in other areas of medicine. For the differential diagnosis of AD, we state that the biomarkers are especially important for the early onset dementias, as there is clinical and radiological overlap, especially between EAD and FTD. In the older age group, the prevalence of AD is much higher, and the usefulness of biomarkers to distinguish AD from other types of dementia becomes less relevant. However, since the currently available medications to enhance cognition are approved for mild to moderate AD, every hint to the correct diagnosis should be taken into account irrespective of age. The additive value of CSF markers to other diagnostic tools has not yet been investigated systematically, and is an aim for future studies.

### **Limitations in research on CSF markers**

For the differentiation of AD from normal aging, depression or other types of dementia overlap is seen in CSF A $\beta$ 42, tau and Ptau concentrations between the groups. One explanation is that the control or demented groups could have neuropathological findings indicative for AD, resulting in an AD biomarker profile. Other explanations are the use of different processing and storage conditions of CSF<sup>71</sup>, the use of different reagent antibodies, differences in the definition of cut off values, and intra- and inter-assay variability of the assays used.<sup>17</sup> Standardization of the (pre-) analytical methods will increase the reliability of the results and it will improve collaboration with other neurological/biochemical research centers or memory clinics. Although it is not difficult to obtain CSF by lumbar puncture, this method is considered to be somewhat invasive for an outpatient clinic, especially in the US. Therefore, a sensitive serum or plasma marker for AD would be very valuable for the use in clinical practice.

### **Conclusion**

For the differentiation of AD from normal aging, depression, or alcoholic dementia the combination of CSF A $\beta$ 42 with tau gives a high sensitivity and specificity of  $\geq 85\%$ , with minimal overlap in individual cases. In the pre-clinical (MCI) stage of disease CSF A $\beta$ 42, tau and Ptau could be used as predictors for the development of AD. For the differentiation of AD from other types of dementia the combination of CSF A $\beta$ 42, tau and Ptau gives a good sensitivity and a reasonable specificity, especially for the differentiation of AD from FTD and less for AD versus DLB or VAD. For clinical practice a high positive predictive value, and a low negative predictive value are important. With at least two markers positive, the diagnosis AD is very likely, while two markers negative can practically rule out the diagnosis AD. The CSF biomarkers must be only used in combination with other diagnostic tools, including clinical investigation, imaging and neuropsychological work up.

### *Guide lines for the use of CSF A $\beta$ 42, tau and Ptau in clinical practice*

1. When there is doubt about the diagnosis AD, with non-conclusive MRI and neuropsychological findings.
2. In patients with early onset dementias (disease onset before 65 years old), as the differential diagnosis here is wider and more complicated; especially the differentiation of EAD from FTD is relevant.
3. In patients suspected for AD, and for whom treatment is being considered.
4. In patients suspected for CJD, in combination with CSF 14-3-3 protein, MRI scan and EEG.

### *Topics for future research*

- Investigate the additional value of the biomarkers CSF A $\beta$ 42, tau and Ptau to other diagnostic methods, i.e. MRI parameters, and/or neuropsychological examinations.
- Investigate the diagnostic value of the biomarkers in primary and secondary referral settings, preferably with neuropathological or prolonged clinical follow-up.
- Investigate which markers could be used for tracking the progression of the disease, especially in the MCI stage of disease. Promising markers are: C- and N-terminally truncated A $\beta$  peptides, oxidative stress markers or inflammatory markers.
- Develop new tests for a sensitive marker, which can be determined in blood or urine.
- Standardize (pre-analytical) laboratory methods between research centers.

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## **CHAPTER 2**

### **AIM OF THE THESIS**



In **chapter 1.2** an overview was given of studies on CSF markers published in the past 10 years. The number of centers investigating biomarkers in AD is increasing, and one would expect that CSF A $\beta$ 42, tau and Ptau will soon be accepted as established diagnostic markers in clinical practice. However, several issues still need thorough attention before this can be done. First, CSF data with neuropathological confirmation of clinically diagnosed AD patients as well as patients with other types of dementia are merely lacking. One must also bear in mind that the exact etiology of AD is still not known. Plaques and tangles are classical in AD, but what triggers the development of senile plaques containing insoluble A $\beta$ 42? Are A $\beta$  peptides indeed the first manifestation of AD –described in the amyloid beta cascade hypothesis<sup>17</sup>- or do they need neurofibrillary tangles at a very early stage of disease to become neuropathogenic, leading to neuron loss and thus clinical symptoms? Recent insights show that various truncated forms and oligomers of A $\beta$  play an important role in disease development<sup>18</sup>, as well as inflammation and oxidative stress.<sup>19</sup> The emerging knowledge from basic science is a must for research on biomarkers, especially for how and when to use them properly in clinical practice.

### **General aim of the thesis**

The aim of studies described in the current thesis was to investigate whether the CSF markers A $\beta$ 42, tau and Ptau can be used for an early and differential diagnosis of AD in clinical practice.

### **Reliability of the assays**

First we had to define whether the assays we (and most centers in Europe) used were reliable with regard to (pre-) analytical factors involved. Among centers there is a large variation of CSF concentrations of A $\beta$ 42 and tau, even when using the same assays in clinically well defined control groups and patients. The manufacturers of the commercial available assays reported that repeated freeze/thaw cycles and tube type may influence concentrations of CSF A $\beta$ 42.<sup>20</sup> Little is known about the stability of the samples when stored for a longer period. We, therefore, addressed the following questions.

*Are CSF samples stable as far as A $\beta$ 42 and tau are concerned?*

We conducted an independent study to investigate the effects of storage temperature, repeated freeze/thaw cycles and centrifugation on CSF concentrations of A $\beta$ 42 and tau (**chapter 3.2**).

This study should shed some light to the influence of pre-analytical factors and their physicochemical mechanisms on CSF concentrations of both markers. Final aim was to formulate standardized conditions, which are crucial for the implementation of the CSF markers as a diagnostic tool in clinical practice.

*Are CSF concentrations of A $\beta$ 42 comparable when measured by two different ELISAs?*

CSF concentrations of A $\beta$ 42 also varied between assays, from decreased levels in AD to no change (two studies) or even increased (one study) levels as compared to controls.<sup>21,22</sup> This might be due to the use of different antibodies directed against distinct epitopes of the A $\beta$  peptide. To address this, we compared CSF concentrations of A $\beta$ 42 as measured by two different A $\beta$ 42 assays in the same CSF samples (**chapter 3.3**).

In addition to N-terminally truncated A $\beta$ 42 peptides various C-terminally truncated A $\beta$  peptides can be found in brain tissue and CSF.<sup>23</sup>

*Is there a relation between various C-terminally truncated A $\beta$  peptides in CSF?*

We wondered whether there was a relation between the three C-terminally truncated A $\beta$  species, A $\beta$ 38, 40 and 42, measured by ELISA in CSF from AD patients and controls (**chapter 4**). By studying the relation between A $\beta$ 42 to other A $\beta$  peptides, we hoped to get a better understanding of the specific decrease of concentration of A $\beta$ 42 in CSF from AD patients, for which the exact mechanism is not known.

### **CSF A $\beta$ 42, tau and Ptau in early and differential diagnosis**

After these more fundamentally orientated studies, we conducted three case-control studies in order to approach the main question: can these CSF biomarkers be used for early and differential diagnosis in clinical practice? To this end several subquestions were formulated, i.e.:

*What is the diagnostic value of CSF A $\beta$ 42, tau and Ptau in early onset AD versus frontotemporal dementia?*

This study (**chapter 5.1**) is particularly relevant as these two types of presenile dementia syndromes show clinical, neuropsychological and radiological overlap. Most previous studies compared older AD patients with younger patients with frontotemporal lobar degeneration

(FTD), or older FTD patients with age-matched AD patients, but in this older age group FTD is less prevalent.<sup>24,25</sup> The combination of CSF A $\beta$ 42, tau and Ptau had not yet been studied in these two types of presenile dementia (see also *chapter 5.2*).

*Are CSF A $\beta$ 42 and tau independent predictors of AD?*

Patients with mild cognitive impairment (MCI) have an increased risk of developing AD, and several studies have shown that CSF A $\beta$ 42 is decreased while CSF tau is increased in MCI.<sup>26,27</sup> We investigated whether MCI patients at different risk for AD according to their biomarker profile differed from each other with respect to other clinical markers or risk factors (*chapter 6*). Furthermore, the relation between CSF A $\beta$ 42 and tau with other clinical markers –medial temporal lobe atrophy (MTA) on MRI scan and memory disturbance- was investigated. To date, cognitive and intellectual impairment with the support of MRI markers are the clinical indicators of AD.<sup>28</sup> CSF biomarkers might well add to this panel of disease markers.

*What is the relation between CSF markers and medial temporal lobe atrophy?*

The final study as reported in this thesis focused on the relation between CSF A $\beta$ 42, tau and Ptau with MTA in a cohort of AD patients and controls (*chapter 7*). The aim of this study was two-fold: first, to get insight whether both CSF and MRI markers reflect the same neuropathological substrate simultaneously. Second aim was to investigate whether both disease markers contribute equally to the diagnosis AD. The comparison with other diagnostic methods becomes more and more important, because neuropathological ante-mortem data as gold standard are missing and none of the disease markers can be 100 percent accurate.

With the abovementioned cross-sectional studies we might well get more insight in whether, how, and when to use the currently most specific biomarkers for AD in clinical practice. Of course much more research is needed, and fortunately enough the topic of biomarkers is an expanding area, especially considering the development of novel biomarkers for diagnosis as well as for tracking progression of the disease.



## **CHAPTER 3**

### **RELIABILITY OF THE ASSAYS**





### **3.1 Description of ELISA's for A $\beta$ 1-42, A $\beta$ N-42, A $\beta$ 38, A $\beta$ 40, total tau and tau phosphorylated at threonine 181**

**- INNOTEST<sup>TM</sup>  $\beta$ -amyloid [1-42] (Innogenetics, Ghent, Belgium)**

The monoclonal antibody (mAb) 21 F12 binds the COOH terminus of the A $\beta$ 42 peptide (amino acids 36-40) and is used as capture antibody. Biotinylated mAb 3D6, which binds the NH2 terminus (amino acids 1-6), is used as detector antibody. Synthetic A $\beta$  (1-42) peptides from Bachem were used as calibrators.

**- Sandwich ELISA for A $\beta$  N-42 (P. Mehta, Staten Island, NY, US)**

Monoclonal antibody 6E10 (Signet Labs) is used as capture antibody, and is specific to an epitope covering N-terminal amino acid residues 1-17. The polyclonal antibody R165 is used as detector antibody. R165 is made by immunizing rabbits with conjugated 33-42 A $\beta$  peptides (Ana Spec). A $\beta$  (1-42) peptides from Bachem were used as calibrators.

**- Sandwich ELISA for A $\beta$ 38 (P. Mehta, Staten Island, NY, US)**

Monoclonal antibody 6E10 (Signet Labs) is used as capture antibody. Antisera to A $\beta$ 38 peptide were produced in rabbits by immunization of peptide "hCys-aminohexanooyl-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-GlyOH". The antibody lacked reactivity against A $\beta$ 40 or A $\beta$ 42 peptides as examined by sandwich ELISA and Western blot analysis. This antibody is used as detector antibody. A $\beta$  (1-42) peptides from Bachem were used as calibrators.

**- Sandwich ELISA for A $\beta$ 40 (P. Mehta, Staten Island, NY, US)**

Monoclonal antibody 6E10 (Signet Labs) is used as capture antibody. The polyclonal antibody R208 is used as detector antibody. R208 is made by immunizing rabbits with conjugated 33-40 A $\beta$  peptides (Ana Spec). A $\beta$  (1-42) peptides from Bachem were used as calibrators.

**- INNOTEST<sup>TM</sup> hTAU-Ag (Innogenetics, Ghent, Belgium)**

Monoclonal antibody AT120 is used as capture antibody, while two mAbs are used as detection antibodies: HT7 and BT2, recognizing different epitopes on the tau protein.

- INNOTEST™ Phosphotau<sub>(181P)</sub> (Innogenetics, Ghent, Belgium)

Monoclonal antibody HT7 is used as capture antibody, and biotinylated mAb AT270 is used as detector antibody, which is specific for the phosphotau-Thr181 epitope.

## **CHAPTER 3.2**

**Effects of processing and storage conditions on CSF amyloid  $\beta$  (1-42) and tau concentrations in cerebrospinal fluid: implications for use in clinical practice**

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*Clin Chem 2005;51:189-195.*

## **Abstract**

*Background:* Reported concentrations of A $\beta$ 42 and tau in cerebrospinal fluid (CSF) differ among reports. We investigated the effects of storage temperature, repeated freeze/thaw cycles, and centrifugation on the CSF A $\beta$ 42 and tau concentrations.

*Methods:* Stability of samples stored at -80°C was determined by use of an accelerated stability testing protocol according to the Arrhenius equation. CSF A $\beta$ 42 and tau concentrations were measured in CSF samples stored at 4°C, 18°C, 37°C and -80°C. Relative CSF concentrations (%) of the biomarkers after 1 freeze/thaw cycle were compared with those after 2, 3, 4, 5, and 6 freeze/thaw cycles. In addition, relative A $\beta$ 42 and tau concentrations in samples not centrifuged were compared to samples centrifuged after 1, 4, 48, and 72 hours.

*Results:* A $\beta$ 42 and tau concentrations were stable in CSF when stored for a long period at -80°C. CSF A $\beta$ 42 decreased by 20% during the first two days at 4°C, 18°C, and 37°C compared with -80°C. CSF tau decreased after storage for 12 days at 37°C. After 3 freeze/thaw cycles CSF A $\beta$ 42 decreased with 20%. CSF tau was stable up to 6 freeze/thaw cycles. Centrifugation did not influence the biomarker concentrations.

*Conclusions:* Repeated freeze/thaw cycles and storage at 4°C, 18°C, and 37°C influence the quantitative result of the A $\beta$ 42 test. Preferably, samples should be stored immediately at -80°C after collection.

## Introduction

In the last decade many studies have set out to find an appropriate biochemical marker for the diagnosis of Alzheimer's disease (AD). Several authors have shown that the sensitivity and specificity of amyloid  $\beta$  (1-42) (A $\beta$ 42) and total tau (tau) in cerebrospinal fluid (CSF) are high when comparing AD patients with controls.<sup>1,2</sup> However, upon comparing AD with other types of dementia<sup>3</sup>, overlap in each biomarker occurs, hampering clinical utility. Ideally, the diagnostic value of biomarkers needs to be validated in neuropathologically confirmed cases, but most studies use the clinical criteria as the gold standard, with risk of circular reasoning. Furthermore, the use of the markers in clinical practice still needs to be established, as most studies have been carried out in research settings with selected patient samples.<sup>4</sup> A recent meta-analysis<sup>5</sup> demonstrated considerable variability in absolute levels of both markers among centres, even when using the same commercial assay. This variability could be attributed to differences in patient groups or to a difference in processing and storage methods among centres. Few published studies have investigated which factors produce a major influence on the quantitative outcome of the INNOTEST™  $\beta$ -amyloid (1-42) ELISA.<sup>6,7</sup> An important confounding factor is the tendency of both A $\beta$ 42 and tau to adhere to glass or hard plastic tubes<sup>4</sup>, reducing the concentration. Furthermore, repeated freeze/thaw cycles seem to play a role in the decrease of CSF A $\beta$ 42, although different methods are used to investigate this phenomenon. One study<sup>6</sup> showed a large decrease of CSF A $\beta$ 42 between the first and second freeze/thaw cycle, while no difference was found between A $\beta$ 42 concentrations in fresh CSF and CSF that had been frozen and thawed once.<sup>7</sup> No studies have been published regarding the stability of both A $\beta$ 42 and tau in CSF when stored frozen at -20°C or -80°C for many years. Knowing sample stability at freezing temperature is especially important for longitudinal studies in which samples are stored for long periods and analyzed simultaneously with samples stored for short periods to minimize inter-assay variability.

In this study we sought to answer the following questions: What are the stabilities of A $\beta$ 42 and tau in CSF samples stored at -80°C for several years? What are the stabilities of A $\beta$ 42 and tau in samples stored at 4°C, 18°C (room temperature), and 37°C up to three weeks, in order to investigate the effect of mailing? What is the effect of repeated freeze/thaw cycles on the concentration of A $\beta$ 42 and tau in CSF? What is the effect of centrifugation on CSF A $\beta$ 42 and tau concentrations? Awareness of pre-analytical factors that may influence the concentration of the markers could improve collaboration with other neurological research centers or

memory clinics and provide more reliable results. Our final aim is to formulate standardized conditions, which will be crucial when the use of A $\beta$ 42 and tau become standard practice for the (early) diagnosis of AD.

## **Materials and Methods**

### *Participants*

Twenty-three individuals provided CSF for the entire study: 3 AD patients, 5 patients with mild cognitive impairment (MCI), 5 patients with frontotemporal dementia (FTD), 1 patient with mixed-type dementia (MD), and 9 controls with no dementia. All individuals gave informed consent to participate in the study. Four patients entered the accelerated stability testing protocol. For the analysis of tau in this experiment, one sample was excluded because the results were higher than the values for the highest calibrator. Two of the four individuals used in the accelerated stability testing protocol also participated in the freeze/thaw experiment. Thirteen other individuals provided CSF for the freeze/thaw experiments: 5 for the comparison of unfrozen CSF versus CSF frozen and thawed once, plus 8 for the comparison of samples frozen and thawed once with samples subjected to several freeze/thaw cycles. Six individuals provided CSF for the centrifugation experiment, including one who provided a haemolytic CSF specimen, which was not centrifuged and was compared with the baseline centrifuged specimen.

### **Lumbar puncture**

CSF was obtained by lumbar puncture in the L3/L4 or L4/L5 intervertebral space, using a 25-gauge needle, and was collected in 12 mL polypropylene tubes. A small amount of CSF was used for routine analysis, including total cells, total protein, and erythrocytes. Within two hours after collection CSF samples were centrifuged at 2100g for 10 minutes at 4°C. Samples were kept at room temperature until centrifugation. After centrifugation, CSF was pipetted into polypropylene tubes in 0.11-, 0.2- or 0.5-mL aliquots, depending on the experiment for which the CSF was to be used.

### *Accelerated stability testing protocol*

For studying the stability at -80°C we used an accelerated stability testing protocol based on the principle of the Arrhenius equation<sup>8</sup>, describing a linear relationship between the logarithm of the reaction rate constant (e.g. the degradation rate) and the inverse of the

absolute temperature. Three temperatures 4°C, 18°C and 37°C were used for calculation of the rate constant.

The principle and calculations of this protocol applying the Arrhenius method are provided in the APPENDIX at the end of this article.

### *Participants*

Two patients and two controls participated in the accelerated stability testing protocol. One patient was a 73-year-old male with MD, and the other a 54-year-old female with probable AD according to the clinical criteria.<sup>9</sup> The controls were two non-demented spouses of patients, a male and a female of 77 and 58 years old.

### *Samples*

After centrifugation, CSF samples were divided into 0.2 and 0.5 mL aliquots. The 0.5 mL aliquot was stored immediately at -80°C (193K) to determine the baseline values for A $\beta$ 42 and tau. The other thirty aliquots of 0.2 mL from each patient were stored at 4°C (277K), room temperature (18°C (291K)) and 37°C (310K), 10 (polypropylene) tubes at each temperature. After 1, 2, and 3 days up to 22 days one tube stored at each of the three different temperatures was removed and frozen at -80°C until analysis. All 30 samples from each patient were thawed and analyzed, in duplicate, simultaneously in one run.

### *Freeze/thaw cycles*

To compare unfrozen CSF with CSF frozen and thawed once, we stored two polypropylene aliquots of 0.2 mL CSF from five individuals for two days either at 4°C or at -80°C until analysis. The concentrations of A $\beta$ 42 and tau in the aliquots that had not been frozen and thawed (stored at 4°C) were compared with the concentrations in the aliquots that had been thawed once (stored at -80°C). All aliquots were tested in duplicate.

As most samples are stored at -80°C until analysis, the best way to simulate daily practice is to compare samples subjected to one freeze/thaw cycle with samples that have undergone several freeze/thaw cycles. Therefore, CSF of 10 individuals was centrifuged and aliquoted into six portions of 0.11 mL. One (polypropylene) tube from each patient was kept at -80°C until analysis and the concentration of A $\beta$ 42 and tau in this aliquot was used as baseline value (100%). The other five (polypropylene) tubes from each patient were stored at -80°C and thawed 2, 3, 4, 5, or 6 times at room temperature for 2 hours and stored again at -80°C until



analysis. The relative A $\beta$ 42 and tau concentrations of the 10 patients (%) were compared with the baseline value (100%) and plotted against the number of freeze/thaw cycles.

#### *Influence of centrifugation*

CSF from five individuals was aliquoted in five 0.5 mL polypropylene tubes. Tube 1 was centrifuged at 2100g for 10 minutes at 4°C within 2h after CSF collection and stored immediately at -80°C. The concentrations of A $\beta$ 42 and tau determined in tube 1 were used as baseline value. Tubes 2, 3, and 4 were stored at 4°C and centrifuged after 4, 48, and 72 hours. After centrifugation the supernatant was pipetted into polypropylene tubes and stored frozen for maximal 1 month until analysis. Tubes 5 were not centrifuged at all and kept for 4 days at 4°C before storage at freezing temperature. Relative A $\beta$ 42 and tau concentrations (%) in samples not centrifuged were compared to samples centrifuged after 1, 4, 48 and 72 hours. In addition, we compared a haemolytic CSF sample obtained after a traumatic lumbar puncture (28,800 erythrocytes/ $\mu$ L, equivalent to approximately 0.5% whole-blood contamination), that was not centrifuged but had been stored at -80°C, with a sample from the same patient centrifuged within 2 hours and stored at 4°C until analysis.

#### *Analysis of A $\beta$ 42 and tau*

A $\beta$ 42 concentrations for all experiments were determined with the sandwich ELISA INNOTEST  $\beta$ -amyloid (1-42) (Innogenetics, Ghent, Belgium). Monoclonal antibody (Mab) 21F12, which is highly specific for the C-terminus of the A $\beta$ 42 peptide, was used as capturing antibody, and the biotinylated Mab 3D6, specific for the N-terminus, was used as detector antibody.<sup>6</sup> For quantification of tau we used the sandwich ELISA INNOTEST<sup>TM</sup> hTau Antigen (Innogenetics) constructed to measure both total tau and phosphorylated tau with Mab AT120 as capturing antibody and HT7 and BT2 as detection antibodies.<sup>10</sup> For the stability experiment, performed at Innogenetics, the mean intra-assay coefficients of variation (CVs) were calculated from the difference between duplicate measurements. The mean CVs for A $\beta$ 42 were 6.2% at an A $\beta$ 42 level of  $\leq$  500 pg/ml (N=61) and 7.2% at an A $\beta$ 42 level of  $>$  500 pg/ml (N=59). For tau the mean intra-assay CV's were 8.7% (tau  $\leq$  300 pg/ml, N=61) and 13.3% (tau  $>$  300 pg/ml, N=26). The mean CV's at the VUMC laboratory were calculated by the precision from the difference between duplicate measurements (SDx100/mean) of 60 routine samples. For A $\beta$ 42 mean CV was 4.0% at concentrations in the low range (125-300 pg/ml), 2.9% at concentrations in the middle range (600-800 pg/ml), and 3.4% at

concentrations in the high range (1000-2000 pg/ml). For tau the CVs were 6.5% at low concentrations (75-200 pg/ml), 4.7% at concentrations in the middle range (500-700 pg/ml) and 4.6% at a high concentration (900-1200 pg/ml). The mean inter-assay CVs of 3 different pools, evaluated in advance and tested in the stability, centrifugation and freeze/thaw experiments, were 12.1% for A $\beta$ 42 (N=7) and 8.1% for tau (N=7). Mean recoveries from four samples to which 1:1 dilutions of the highest calibrators for A $\beta$ 42 or tau were added were 77% (range 73%-81%) for A $\beta$ 42 and 109% (range 108%-112%) for tau.

## Results

### *Stability at -80°C according to the Arrhenius equation*

The relative concentrations (%) of A $\beta$ 42 and tau in CSF from the four and three patients on the consecutive days were calculated at 4°C, 18°C and 37°C, with the baseline value (-80°C) set at 100%. Plotting the relative concentrations (%) versus the days of heat stress, the rate constants at each investigated temperature (k(T)) were calculated (Table 1). The calculated k(T) values, determined at the three temperatures, were not different from zero, indicating that A $\beta$ 42 and tau are stable in CSF when stored at -80°C.

**Table 1 Rate constants (k(T)) for A $\beta$ 42 and tau**

| <u>Temperature (K)</u> | <u>k(T) of A<math>\beta</math>42</u> | <u>k(T) of tau</u> |
|------------------------|--------------------------------------|--------------------|
| 277                    | -0.0043 ( $\pm$ 0.04)                | 0.0018 (0.03)      |
| 291                    | -0.0032 (0.03)                       | -0.0016 (0.03)     |
| 310                    | 0.050 (0.03)                         | -0.039 (0.03)      |

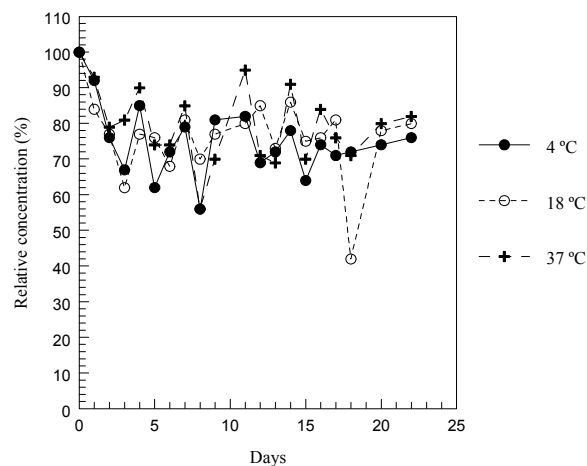
Percentages of remaining measurable protein were plotted versus days of heat stress at each temperature. Mean (SD) k values were determined for the three temperatures at the slope of the best-fit line.

### *Stability during mailing conditions*

When we plotted the mean CSF A $\beta$ 42 values for samples from four patients stored at the three different temperatures against time, the protein concentrations were highest at baseline (-80°C) and during the first two days, the concentrations decreased by 20% in samples stored at 4°C, 18°C and 37°C (Figure 1A). Thereafter, the concentration of CSF A $\beta$ 42 remained

**Figure 1 Relative concentrations of Aβ42 in CSF samples stored at 4°C, 18°C and 37°C**

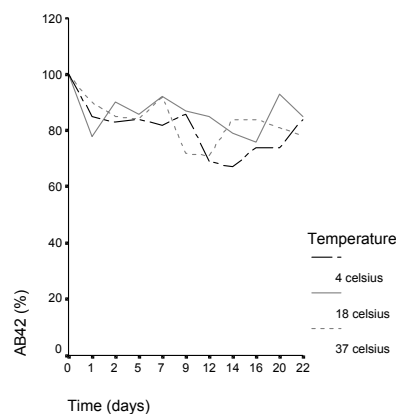
**Figure 1A**



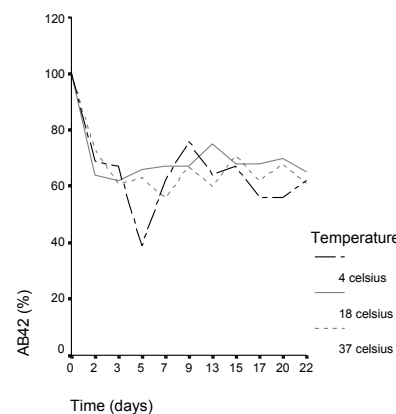
Mean percentages of Aβ42 plotted vs time, in samples collected from four subjects, and stored at 4°C, 18°C and 37°C compared with the baseline sample, stored at -80°C and stated as 100%.

**Figure 1B**

**Subject 1:**



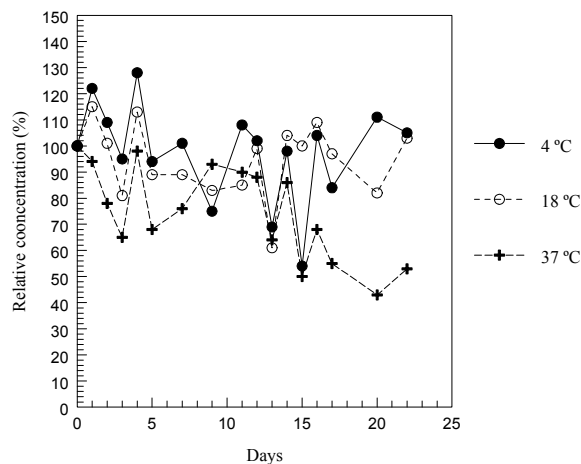
**Subject 2:**



Percentages of Aβ42 plotted vs time in CSF samples from subject 1 (mixed type dementia) and subject 2 (control) and stored at 4°C, 18°C and 37°C

relatively stable up to 22 days, although we observed considerable variability between aliquots from the same individual (Figure 1B). Tau plots showed that the protein was stable in CSF at 4°C and 18°C, whereas the concentration decreased at 37°C after approximately 12 days (Figure 2).

**Figure 2 Relative concentrations of tau in CSF samples stored at 4°C, 18°C and 37°C**



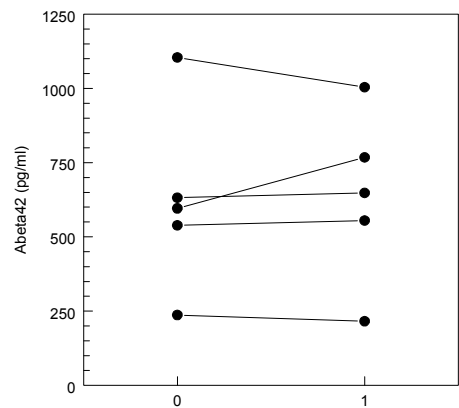
Mean percentages of tau, plotted vs time, in CSF samples collected from three patients and stored at 4°C, 18°C and 37°C compared with baseline sample, stored at -80°C and stated as 100%.

### *Freeze/thaw cycles*

No difference could be demonstrated between concentrations of A $\beta$ 42 and tau in CSF that had not been thawed and CSF that had undergone one freeze/thaw cycle (visualized for A $\beta$ 42 in Figure 3A). In Figure 3B the mean (SD) relative concentrations (%) for A $\beta$ 42 vs the number of freeze/thaw cycles are shown. A decrease of 20% after 3 freeze/thaw cycles was observed. Thereafter, no decline could be demonstrated and the values remained constant at 80% of the baseline concentration during 6 freeze/thaw cycles. The change in concentration varied among individuals, ranging from no change in the samples from one patient to a large decrease in samples from another. We could not demonstrate a difference between samples with high concentrations of A $\beta$ 42 ( $\geq 550$  pg/ml, n=5) and low concentrations of A $\beta$ 42 (<550 pg/ml, n=5). In Figure 3C the mean (SD) relative concentrations (%) of tau vs the number of freeze/thaw cycles are shown. No change during 6 freeze/thaw cycles could be demonstrated.

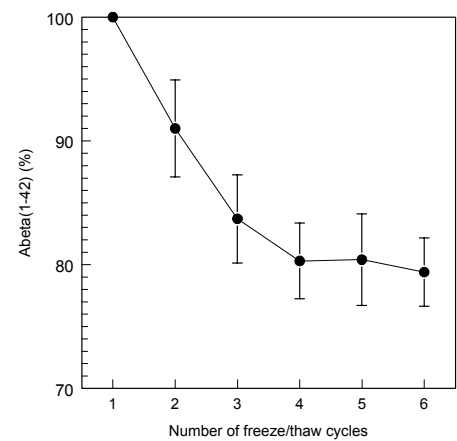
**Figure 3 Effect of freezing/thawing on Aβ42 and tau concentrations**

**Figure 3A**



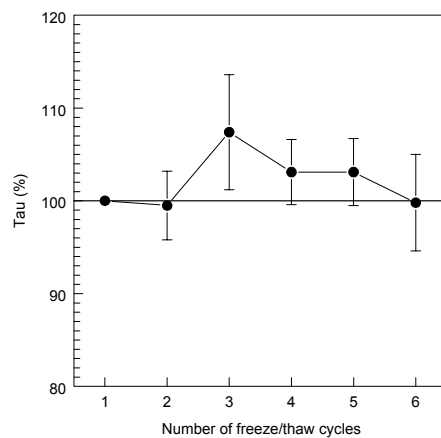
Plot of CSF Aβ42 concentration in five samples that had not been subjected to a freeze/thaw cycle compared with samples that had been subjected to one freeze/thaw cycle.

**Figure 3B**



Mean (SD, error bars) relative CSF Aβ42 concentrations (%) in samples of ten patients versus numbers of freeze/thaw cycles. The values of Aβ42 after 1 freeze/thaw cycle of each subject were used as baseline value and stated as 100%.

**Figure 3C**



Mean (SD, error bars) relative CSF tau concentrations (%) in samples of nine patients versus numbers of freeze/thaw cycles. The values of tau of each subject after 1 freeze/thaw cycle were used as baseline value and stated as 100%.

#### *Influence of centrifugation*

No difference was found between CSF concentrations of A $\beta$ 42 and tau in samples that were stored at 4°C and centrifuged after 1, 4, 48, or 72 hours. Furthermore, there was no difference in concentrations of the markers in samples stored frozen after centrifugation, and samples that were not centrifuged and stored for 4 days at 4°C. In addition, no difference in A $\beta$ 42 and tau concentrations could be shown in the haemolytic sample not centrifuged and stored at -80°C (A $\beta$ 42 662 ng/L; tau 232 ng/L) and the sample centrifuged and stored at 4°C (A $\beta$ 42 = 596 ng/L; tau = 230 ng/L).

#### **Discussion**

Using the Arrhenius approach, we showed that the A $\beta$ 42 and tau concentrations are stable in CSF samples when frozen immediately and stored for a longer period at -80°C. Furthermore, the concentration of A $\beta$ 42 in CSF decreased by approximately 20% during the first two days when stored at 4°C, 18°C and 37°C compared to the baseline value, and then remained constant for up to 22 days, although with considerable variability between aliquots from the same individual. CSF tau concentration was stable at 4°C and 18°C, but showed a decrease

after 12 days when stored at 37°C. After 4 freeze/thaw cycles the concentration of A $\beta$ 42 in CSF decreased by 20%, while tau remained stable during 6 freeze/thaw cycles. Centrifugation did not influence the outcome of either biomarker.

To the best of our knowledge, the stability of A $\beta$ 42 and tau in CSF samples stored at -80°C for many years has never been systematically investigated. Two previous studies found that CSF A $\beta$ 42<sup>2,5</sup> and tau<sup>5</sup> concentrations remained stable when stored for > 6 months at -70°C or -80°C. The first study showed that the correlation between CSF A $\beta$ 42 measured at different times during 1 year and reanalysis at one time was high (correlation coefficient = 0.96).<sup>2</sup> Unfortunately, the regression coefficient (or slope of the line) is not mentioned in that study, while this could give more accurate information about degradation of the protein. The second study did not find a relation between CSF A $\beta$ 42 or tau and shelf life.<sup>5</sup> In our study we investigated the long-term stability at -80°C with an accelerated stability testing protocol according to the Arrhenius method. No significant decline in A $\beta$ 42 and tau concentration at 4°C, 18°C, and 37°C was found, except for tau at 37°C, and only after 12 days. Therefore, the degradation constant was not different from zero, and no Arrhenius-plot or projected stability time could be calculated. From this we conclude that both proteins are very stable in CSF and that the samples can be stored for a very long period at -80°C. However, a real-time stability experiment performed in the future is needed to confirm our data.

The stability of various forms of A $\beta$  in CSF samples stored at different temperatures has been described in two studies<sup>11,12</sup> using in-house ELISAs. In the first study<sup>11</sup>, the immunoreactivity of CSF A $\beta$ 40 and A $\beta$ 42 decreased by 8% and 10% when kept for 24 hours at 20°C, but remained stable the first 24 hours at 4°C. In the second study<sup>12</sup>, CSF total A $\beta$  levels were measured and found to be unstable if samples were stored at -20°C, 4°C, and room temperature, with the largest decrease during the first day and plateauing after the third day. Although different assay formats were used, measuring different types of A $\beta$ , abovementioned findings support our results of a decrease in concentration of CSF A $\beta$ 42 during the first two days for samples stored at 4°C, 18°C and 37°C. Although incubation at higher temperatures could have an effect on the binding capacity of A $\beta$ 42<sup>6,13,14</sup>, storage of CSF at different temperatures does not seem to affect the A $\beta$ 42 concentration<sup>6</sup>, which is supported by our findings showing comparable A $\beta$ 42 concentrations in CSF samples stored at 4°C or 37°C. There was a difference in A $\beta$ 42 concentration only between samples stored at -80°C immediately after collection or stored at higher temperatures after collection. An

interesting finding in one study<sup>11</sup> was that the antibody-binding capacity of synthetic A $\beta$ 42 was lower in CSF than in water. In addition, A $\beta$ 42 levels were lower in artificial CSF with physiological concentrations of albumin than without albumin. An explanation for this finding could be that binding of A $\beta$ 42 to albumin masks the epitope recognized by A $\beta$ 42-specific antibodies.<sup>15,16</sup> This binding of A $\beta$ 42 to other proteins might also cause the low recovery rate of the A $\beta$ 42 ELISA, although Vanderstichele et al.<sup>6</sup> and others<sup>17</sup> could not find interference with A $\beta$  by other proteins, including human albumin. However, interference experiments are largely dependent on the protocol being used, and whether pre-incubation is needed. Furthermore, results are also dependent on which medium is used, either artificial CSF, human CSF or another medium such as sample diluent.<sup>6</sup> The difference in concentrations of A $\beta$ 42 between samples stored at  $-80^{\circ}\text{C}$  and at higher temperatures could also be the result of binding of A $\beta$ 42 to other proteins, but conformational changes, aggregation<sup>18</sup> or degradation may be involved as well. The variability in A $\beta$ 42 concentrations among centers<sup>5</sup> might very well be attributable to the procedure for sample treatment the first hours after collection, with one center immediately freezing samples on dry ice and another center storing samples at room temperature until further processing (our center). This is an important factor to be considered and investigated in future multicenter studies.

Our finding of decreased concentrations of A $\beta$ 42 in CSF after repeated freeze/thaw cycles corresponds with the outcome of several other studies<sup>6,11,12</sup>, and stresses again the importance of avoiding freeze/thaw cycles in order to minimize the risk of falsely low A $\beta$ 42 values. The decrease of CSF A $\beta$ 42 after repeated freeze/thaw cycles might also be explained by the same physicochemical mechanisms –i.e. conformational change of the fibrillary  $\beta$ -sheeted A $\beta$ 42 protein or masking of the epitope by binding to other proteins- that led to a decrease of A $\beta$ 42 concentration during the first two days when stored at  $4^{\circ}\text{C}$ . This is sustained by our finding of comparable CSF A $\beta$ 42 concentrations between samples that had not been subjected to a freeze/thaw cycle (stored for two days at  $4^{\circ}\text{C}$ ) versus samples that had been subjected to 1 freeze/thaw cycle.

Tau protein is considered to be very stable, and also repeated freeze/thaw cycles do not seem to influence the concentration of this protein. Therefore, it is remarkable that tau decreases after 12 days storage at  $37^{\circ}\text{C}$ . At this temperature, the protein may be degraded by proteases, form aggregates or undergo conformational changes, producing a form that is not detectable by one or both anti-tau antibodies used in the ELISA.<sup>10</sup> Little is known about the relationship



between CSF tau and temperature. The pathological core protein of paired helical filaments (PHF), consisting of a portion of tau, is found to be protease and heat resistant.<sup>19</sup> However, the aggregation of tau into PHF has been demonstrated to increase at temperatures above 30°C.<sup>20</sup> The tau protein is generally highly soluble, but the aggregated pathological form found in the neurofibrillary tangles in the brain might not be released in CSF. The nature of tau in CSF is not well documented, but previous studies have revealed different molecular masses of tau in lumbar CSF ranging from 25-80 kDa.<sup>21</sup> The low molecular weight of 25 kDa is not found in the brain, suggesting that CSF tau is truncated when released into CSF, probably as a result of degradation processes occurring in the brain. The truncated forms of tau in CSF should be well recognized by the anti-tau antibodies as they cover only a small part of the large full-length tau. We speculate that the 12 days storage of CSF at 37°C might lead to a change of the truncated form of tau into a more aggregated form, which is undetectable by the antibodies incorporated in the ELISA.<sup>10</sup>

In conclusion, both A $\beta$ 42 and tau are stable in CSF at -80°C for a long period. However, CSF A $\beta$ 42, when stored at 4°C, 18°C and 37°C, decreased by 20% during the first two days compared with the baseline value (-80°C). Furthermore, the concentration of A $\beta$ 42 in CSF is influenced by the number of freeze/thaw cycles. To avoid these difficulties it is best to process CSF as soon as possible after collection and store it at -80°C for long storage. Preferably, CSF samples should be sent on dry ice when stored frozen.

## Acknowledgements

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#### *APPENDIX Estimation of stability by the Arrhenius method*

The best way to determine the stability of analytes in body fluids is to perform a real time stability experiment. This can be done by storing paired aliquots of a sample and determining the concentrations of the proteins after certain time points, varying from some months to many years, taking into account that the sample is thawed once. However, this approach requires long experimental periods. Moreover, in real stability time studies, the determination of the protein must be performed at the beginning, during and at the end of the study. These measurements can often not be performed using the same batch of reagents. An increased inter-assay coefficient of variation may be the result.

Therefore, estimation of protein stability should rather be performed using an accelerated stability testing protocol. The kinetics of protein denaturation are comparable with that of a first order reaction, which means that the degradation rate is proportional to the concentration of the respective analyte. The equation for the accelerated stability testing protocol is:

$$-d[C]/dt = k[C] \text{ or } \ln C(t)/C(0) = -k \cdot t$$

where  $C(0)$  is the initial protein concentration,  $C(t)$  is the concentration after time  $t$ , and  $k$  is the rate constant. The rate constant, which is dependent of the temperature, is determined at the three fixed temperatures 4°C, 18°C and 37°C, assuming that at -80°C the concentrations remain constant during the time of this storage experiment. Afterwards the Arrhenius equation is applied, using this formula:

$$\ln k(T) = A + E/RT$$

in which  $A$  = the pre-exponential factor and  $E/R$  = the slope of the equation. Using the equation of the best-fit line, it is possible to calculate the degradation rate constant at each desired temperature:

$$\ln k(T) = A + B/T$$

or

$$y = A + Bx$$

$A$  and  $B$  have fixed values. By substituting the temperature of interest, e.g. -80° C = 193°K →  $k(193)$  is calculated. We can calculate the time after which 90% or 95% of Aβ42 or tau can be recovered, by substituting  $C(t)/C(0) = 0.90$  or  $0.95$  in the equation:

$$\ln(0.90) = -k(193) \cdot t$$

$$-0.105 = -k(193) \cdot t$$

$$t = 0.105/k(193)$$

or

$$\ln(0.95) = -k(193) \cdot t$$

$$-0.0513 = -k(193) \cdot t$$

$$t = 0.0513/k(193)$$

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## **CHAPTER 3.3**

### **Differences and similarities between two frequently used assays for A $\beta$ 42 in cerebrospinal fluid**

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## **Abstract**

*Background:* Differences in absolute concentrations and clinical performance of cerebrospinal fluid (CSF) amyloid  $\beta$  42 ( $A\beta$ 42) between laboratories is partly attributable to the antibodies selected for the assay. We compared  $A\beta$ 42 levels and diagnostic accuracy of two  $A\beta$ 42 assays in the same CSF samples.

*Methods:*  $A\beta$ 42 levels were measured in CSF of 39 Alzheimer's disease (AD) patients, 24 patients with frontotemporal lobar degeneration (FTLD) and 30 controls. One ELISA used the monoclonal antibodies 3D6 and 21F12 directed against amino acids 1-6 of the N-terminal part and amino acids 36-42 of the C-terminal part of  $A\beta$ 42 ( $A\beta$  1-42). The other ELISA used the monoclonal antibody 6E10 specific to an epitope present on 1-17 amino acid residues of the N-terminal part and the polyclonal antibody R165 directed against amino acids 33-42 of the C-terminal part of  $A\beta$ 42 ( $A\beta$  N-42).

*Results:* Absolute concentrations of CSF  $A\beta$  1-42 and  $A\beta$  N-42 were comparable in all CSF samples. In AD versus controls sensitivity and specificity values for CSF  $A\beta$  1-42 and  $A\beta$  N-42 were equal;  $A\beta$  1-42: sensitivity 90% and specificity 93%;  $A\beta$  N-42: sensitivity 90% and specificity 87%. A slightly better differentiation of AD from FTLD was obtained with CSF  $A\beta$  N-42 than CSF  $A\beta$  1-42 (area under the ROC curve  $A\beta$  1-42= 0.77, 95%CI 0.64-0.90 and  $A\beta$  N-42= 0.87, 95%CI 0.76-0.97,  $P=0.045$ ).

*Conclusions:* Both  $A\beta$ 42 assays provided equal diagnostic accuracy comparing AD with controls. Further studies are needed to investigate the involvement of the different forms of  $A\beta$ 42 in AD and FTLD patients.

Amyloid  $\beta$  42 (A $\beta$ 42) concentrations in cerebrospinal fluid (CSF) are used to identify Alzheimer disease (AD)<sup>1</sup>, but reported concentrations differ among studies as does diagnostic accuracy.<sup>2</sup> These differences may relate to the patient and control groups (3) studied, processing and storage methods<sup>4</sup>, intra- and inter-assay variation of the assays, or to the reagent antibodies used. A recent meta-analysis<sup>2</sup> stressed the importance of standardizing assays for A $\beta$ 42 in CSF. In most studies CSF A $\beta$ 42 was reported to be decreased, but in two studies, CSF A $\beta$ 42 was not significantly changed in AD<sup>2</sup>, and in one study even increased in the early stages of AD.<sup>5</sup> These dissimilarities might reflect the specificities of the antibodies incorporated in the assays.

The first aim of our study was to compare levels of A $\beta$ 42, as measured by two different assays in the same CSF samples. The first assay, widely used in Europe<sup>6</sup>, uses two monoclonal antibodies (Mabs) and detects full length A $\beta$ 42 peptide, A $\beta$  1-42.<sup>7</sup> The second assay [A $\beta$  (N-42)], used mainly in the United States<sup>8</sup>, detects both full length A $\beta$ 42 and A $\beta$  peptides truncated at the N-terminus.<sup>9</sup>

The second aim of the study was to compare diagnostic accuracies of the assays for patients with AD compared with non-demented controls and patients with frontotemporal lobar degeneration (FTLD).

Finally, we investigated the relationship between CSF A $\beta$  1-42 and A $\beta$  N-42 concentrations and albumin ratio, age, disease duration, and disease severity.

Between October 2000 and December 2002 39 AD patients, 24 FTLD patients and 30 non-demented controls were recruited at the Alzheimer Center of the VU University Medical Center (VUMC). All patients underwent a standardized investigative protocol.<sup>3</sup> A diagnosis of 'probable' AD was made according to the NINCDS-ADRDA criteria<sup>10</sup>; the clinical picture of FTLD (including frontotemporal dementia, semantic dementia, and progressive aphasia) was based on international clinical diagnostic criteria.<sup>11</sup> Disease duration in AD and FTLD patients was defined as the time in years between the first symptoms by history and the lumbar puncture.

The control group (n = 30) consisted of 20 persons with subjective memory complaints, who had undergone the same protocol of examinations as the patients; five healthy spouses of patients without memory complaints; three individuals with a positive family history for AD, all without memory complaints; one patient with a suspicion of benign intracranial



hypertension and one patient with a possible neuritis vestibularis. No controls developed dementia or mild cognitive impairment within 1 year. The Mini Mental State Examination (MMSE) score<sup>12</sup> was used as a measure of global cognitive impairment. The study was approved by the ethics review board of the VUMC. All patients and controls gave written informed consent.

CSF was collected and stored as described previously.<sup>4</sup> The albumin ratio (serum albumin/CSF albumin) was used as a measurement of the intactness of the blood-brain barrier. Except for one FTLN patient and 2 controls, the blood-brain barriers of the patients were intact (Table 2).

The INNOTEST<sup>TM</sup>  $\beta$ -AMYLOID<sub>(1-42)</sub> (Innogenetics) uses Mab 21F12, which binds the C-terminus of the A $\beta$ 42 peptide (amino acids 36-42), as capture antibody, and biotinylated Mab 3D6, which binds the N-terminus (amino acids 1-6), as detector antibody (6). A $\beta$  (1-42) peptides from Bachem were used for calibration. To minimize aggregates in the peptide stocks used to prepare calibrators, we obtained three different batches of A $\beta$ 1-42 (Bachem) and processed them together. Peptides were pre-treated to eliminate the occurrence of small oligomers/aggregates. This test was performed at the Department of Clinical Chemistry of the VUMC.

The sandwich ELISA for A $\beta$  N-42 uses the commercially available Mab 6E10, specific to an epitope covering N-terminal amino acid residues 1-17 of A $\beta$ 42 (Signet Labs) as capture antibody, and the polyclonal antibody R165 as detector antibody. R165 was made by immunizing rabbits with conjugated A $\beta$ 33-42 peptides (Ana Spec). A $\beta$ 1-42 from Bachem was used for calibration, although production procedures for the calibrators were slightly different between the two laboratories. This test was performed at the New York site according to an in-house protocol.

For statistical analysis, SPSS version 11.0 was used. Non-parametric statistics were used, as the distribution of the variables was not normal. Passing and Bablok regression<sup>13</sup> was calculated with Medcalc, V 4.30 (Medcalc Software), and we also prepared a Bland and Altman plot.<sup>14</sup> For group differences we applied the Kruskal-Wallis test, followed by the Mann Whitney U test applying the Bonferroni correction. The Chi-square test with continuity correction was used to test group differences within genders.

The sensitivities and specificities for CSF A $\beta$  1-42 and A $\beta$  N-42 were also calculated by Medcalc. Cut points corresponded to a sensitivity  $\geq$  85% (15), but if a higher sensitivity was

obtained for a reasonable specificity, it was used. Receiver operating characteristic (ROC) curves were constructed and compared.<sup>16</sup> Spearman correlations were calculated. A test was considered significant at  $P < 0.05$ . All reported tests are two-tailed unless stated otherwise.

The CSF A $\beta$  1-42 and A $\beta$  N-42 concentrations were not statistically significantly different (Table 1, Figures 1 and 2, see also Appendix and the online version of this Technical Brief at <http://www.clinchem.org/content/vol51/issue 6/>).

**Table 1 Passing and Bablok regression equation**

|              | N  | Equation      | Slope 95% CI | Intercept 95% CI |
|--------------|----|---------------|--------------|------------------|
| AD           | 39 | $Y=0.71X+35$  | 0.51-1.01    | -39 - +106       |
| FTLD         | 24 | $Y=1.17X-39$  | 0.89-1.59    | -230 - +99       |
| Controls     | 30 | $Y=1.36X-245$ | 0.81-2.44    | -947 - +89       |
| All subjects | 93 | $Y=1.12X-67$  | 0.95-1.3     | -140 - -3        |

AD = Alzheimer's disease; FTLD = frontotemporal lobar degeneration. CI = confidence interval. Variable X = A $\beta$  1-42, variable Y = A $\beta$  N-42.

Concentrations of both CSF A $\beta$  1-42 and A $\beta$  N-42 levels were significantly lower in AD patients than in FTLD patients and controls (Table 2).

CSF A $\beta$  1-42 concentrations differed significantly between FTLD patients and controls, whereas CSF A $\beta$  N-42 concentrations did not differ significantly between the two groups (Table 2). The ratio of A $\beta$  1-42 to A $\beta$  N-42 differed significantly only between the AD and FTLD patient groups.

ROC curves for CSF A $\beta$  1-42 and A $\beta$  N-42 are shown in Figure 3. In AD versus controls sensitivity and specificity for CSF A $\beta$  1-42 were 90% and 93% at 473 ng/L, and for CSF A $\beta$  N-42 90% and 87%, respectively, at 383 ng/L. No difference was present in diagnostic accuracy of CSF A $\beta$  1-42 compared to CSF A $\beta$  N-42 (Figure 3A); AUC A $\beta$  1-42 = 0.94, 95%CI 0.86 - 0.99 versus AUC A $\beta$  N-42 = 0.92, 95% CI 0.83 - 0.97,  $P = 0.47$ .

**Table 2 Demographic data and CSF analyses for each diagnostic category**

|                               | AD<br>(N=39)   | FTLD<br>(N=24) | Co + OND<br>(N=30) | AD vs FTLD<br>(P-value) | AD vs controls<br>(P-value) | FTLD vs controls<br>(P-value) |
|-------------------------------|----------------|----------------|--------------------|-------------------------|-----------------------------|-------------------------------|
| Age (yrs)                     | 62 (52-79)     | 63 (49-85)     | 64 (32-79)         | 0.58                    | 0.14                        | 0.66                          |
| Sex (M/F)                     | 20/19          | 16/8           | 14/16              | 0.26                    | 0.90                        | 0.41                          |
| Duration (yrs)                | 4 (1-11)       | 3 (1-11)       | --                 | 0.054                   | --                          | --                            |
| MMSE                          | 20 (3-28)      | 24 (3-29)      | 30 (25-30)         | 0.02                    | <0.001                      | <0.001                        |
| Albumin ratio                 | 4.8 (2.0-10.6) | 5.3 (1.5-17.3) | 5.2 (2.8-18.5)     | 0.6                     | 0.47                        | 0.99                          |
| A $\beta$ 1-42 (pg/mL)        | 315 (140-626)  | 495 (202-1087) | 651 (337-1224)     | <0.001                  | <0.001                      | 0.02                          |
| A $\beta$ N-42 (pg/mL)        | 288 (116-674)  | 588 (150-1324) | 629 (218-1075)     | <0.001                  | <0.001                      | 0.66                          |
| A $\beta$ 1-42/A $\beta$ N-42 | 1.1 (0.5-1.7)  | 0.9 (0.4-1.3)  | 1.0 (0.6-2.6)      | 0.001                   | 0.24                        | 0.07                          |

AD = Alzheimer's disease; FTLD = frontotemporal lobar degeneration; Co = controls; yrs = years; M/F = Male/Female; MMSE = Mini Mental State Examination;  $\geq 1$   $\epsilon 4$  alleles = one or two  $\epsilon 4$  alleles. Values are expressed as medians (minimum-maximum). P-values refer to statistical difference between AD vs FTLD, AD vs controls or FTLD vs controls.

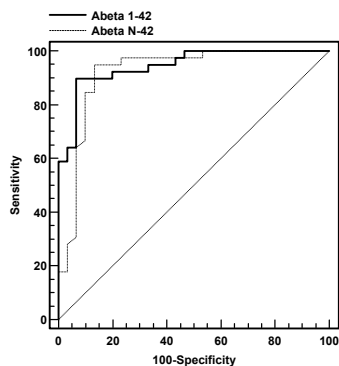
When comparing AD with FTLN, a specificity of 67% was obtained for CSF A $\beta$  1-42 at a sensitivity of 85% (448 ng/L). For CSF A $\beta$  N-42 specificity was 75% at a sensitivity of 87% (373 pg/mL). The AUCs for CSF A $\beta$  N-42 and CSF A $\beta$  1-42 tended to be different (Figure 3B); AUC A $\beta$  1-42= 0.77 (0.64-0.90) and AUC A $\beta$  N-42= 0.87 (0.76-0.97),  $P=0.045$ . The AUCs for CSF A $\beta$  1-42 and CSF A $\beta$  N-42 (Figure 3C) in distinguishing FTLN patients from controls were significantly different [AUC A $\beta$  1-42= 0.69 (0.55-0.81) and AUC A $\beta$  N-42= 0.54 (0.39-0.67),  $P=0.007$ ], but the discriminatory value was small for A $\beta$  1-42 and negligible for A $\beta$  N-42, with the confidence interval for the AUC including 0.5.

We found no significant correlation of either CSF A $\beta$  1-42 or A $\beta$  N-42 with albumin ratio, MMSE, age or disease duration (AD and FTLN) in either group.

The absolute concentrations of CSF A $\beta$  1-42 and A $\beta$  N-42 were comparable. However, in earlier studies concentrations of CSF A $\beta$  N-42 ranged from 36 pg/mL to 623 pg/mL in AD and 111 pg/mL to 629 pg/mL in controls.<sup>8,17,18</sup> The reason for the low CSF A $\beta$  N-42 concentrations measured in these studies could be a difference in the affinity of the A $\beta$  N-42 polyclonal antiserum samples or the purity and solubility of the peptides used as calibrator.<sup>8</sup> The sensitivity of an ELISA depends largely on the binding characteristics of the antigen, which may vary with temperature and buffer solutions, or among different reagent lots.<sup>6</sup> In addition, the affinity of the antibodies used in the assays might vary for the various A $\beta$ 42 peptides involved in the AD pathogenesis, including oligomers of the A $\beta$ 42 peptide. A future study exchanging calibrators and antibodies among various ELISAs is necessary for harmonization. ROC curve analysis revealed no difference in the ability of the two assays to differentiate between AD patients from controls. Next to the C-terminal heterogeneity, various N-terminal truncated peptides are found in the A $\beta$  pools of AD brains.<sup>19,20</sup> These peptides are considered to play a role in the increased A $\beta$ 42 production in developing AD. We speculate that A $\beta$  1-42 and A $\beta$  N-42 concentrations go hand in hand at a certain stage of disease, in mild to moderate AD as well as in controls. Because the N-terminally truncated A $\beta$ 42 peptides can be demonstrated early in the disease process<sup>9</sup>, they might be promising markers for the preclinical diagnosis of AD, when used simultaneously with A $\beta$  1-42.<sup>21</sup>

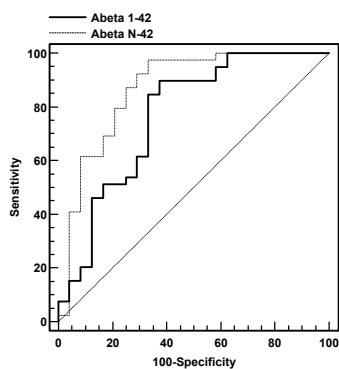
**Figure 3 ROC curve A $\beta$  1-42 versus A $\beta$  N-42**

**3A. AD vs controls**



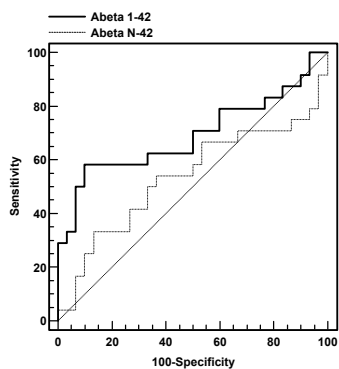
ROC curve comparing A $\beta$  1-42 (straight line) with A $\beta$  N-42 (dotted line) in AD versus controls

**3B. AD vs FTLD**



ROC curve comparing A $\beta$  1-42 (straight line) with A $\beta$  N-42 (dotted line) in AD versus FTLD

**3C. FTLD vs controls**



ROC curve comparing A $\beta$  1-42 (straight line) with A $\beta$  N-42 (dotted line) in FTLD versus controls

The difference in diagnostic accuracy of CSF A $\beta$  1-42 and A $\beta$  N-42 comparing AD with FTLD is remarkable. Several authors found a decrease of A $\beta$  1-42 in CSF in a subset of FTLD patients.<sup>3,22</sup> Hardly any information is available about the CSF A $\beta$  N-42 concentration in FTLD.<sup>17</sup> The reason for a decrease of CSF A $\beta$  1-42 in FTLD is unknown, although there might be a relation with the presence of an Apo E  $\epsilon$ 4 allele or with age.<sup>23</sup> Interestingly, a few studies have shown the involvement of three mutations in the presenilin 1 gene (PSEN1) mutations in familiar forms of FTLD.<sup>24-26</sup> These possible 'loss of function' PSEN1 mutations might act as inhibitors of the  $\gamma$ -secretase cleavage of APP<sup>27</sup>, leading to a decrease of A $\beta$  1-42 in the brain. Although most FTLD patients included in our study have the sporadic form of FTLD, we cannot exclude the possibility of a mutation in the PSEN1 gene in some of them.

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## **CHAPTER 4**

### **AMYLOID $\beta$ 38, 40, AND 42 SPECIES IN CEREBROSPINAL FLUID: MORE OF THE SAME?**

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**Abstract**

Various C-terminally truncated amyloid  $\beta$  peptides ( $A\beta$ ) are linked to Alzheimer's disease (AD) pathogenesis. Cerebrospinal fluid (CSF) concentrations of  $A\beta_{38}$ ,  $A\beta_{40}$ , and  $A\beta_{42}$  were measured by enzyme-linked immunosorbent assay in 30 AD patients and 26 controls. CSF  $A\beta_{42}$  levels were decreased in AD, while CSF  $A\beta_{38}$  and  $A\beta_{40}$  levels were similar in AD and controls. All three  $A\beta$  peptides were inter-related, particularly CSF  $A\beta_{38}$  and  $A\beta_{40}$ . Diagnostic accuracy of CSF  $A\beta_{42}$  was not improved by applying the ratios of CSF  $A\beta_{42}$  to  $A\beta_{38}$  or  $A\beta_{40}$ .

## Introduction

The amyloid  $\beta$  (A $\beta$ ) peptides comprise a heterogeneous set of N- and C-terminally truncated peptides as has been demonstrated in cell culture supernatants, brain tissue and cerebrospinal fluid (CSF).<sup>1-3</sup> The three best known C-terminally truncated A $\beta$  peptides are A $\beta$ 38, A $\beta$ 40 and A $\beta$ 42. A $\beta$ 38 has been found to be the second prominent soluble A $\beta$  peptide species in CSF after A $\beta$ 40.<sup>3</sup> A $\beta$ 42 is decreased in CSF of Alzheimer's disease (AD) patients compared with control subjects.<sup>4,5</sup> There is evidence that soluble A $\beta$  peptides are more related to disease severity in AD than the insoluble A $\beta$ 42 peptide.<sup>3,6</sup> Furthermore, the ratio of A $\beta$ 42 to A $\beta$ 40 is stated to classify more AD patients correctly compared with CSF A $\beta$ 42 alone.<sup>7</sup>

With the emerging strategies for disease modification in AD<sup>8,9</sup>, quantification of other A $\beta$  species, in addition to A $\beta$ 42, linked to AD pathology may gain importance. To evaluate this, we measured CSF concentrations of A $\beta$ 38, A $\beta$ 40, and A $\beta$ 42 by enzyme-linked immunosorbent assays in AD and control subjects. The relations between the A $\beta$  species and their correlations to clinical variables were also investigated. Finally, the diagnostic accuracy of CSF A $\beta$ 42 in AD versus controls subjects was compared with that of the ratios of CSF A $\beta$ 42 to A $\beta$ 40 and CSF A $\beta$ 42 to A $\beta$ 38.

## Subjects and methods

### *Subjects*

Thirty AD patients and 26 controls were recruited at the Alzheimer Center of the VUMC. All AD patients underwent a standardized investigative protocol as described in detail previously.<sup>5</sup> Diagnosis of probable AD was made according to the NINCDS-ADRDA criteria.<sup>10</sup> The control group consisted of 20 subjects with subjective memory complaints, two subjects with a positive family history and four healthy spouses without memory complaints. None of the controls experienced development of dementia within a follow-up period of one year. The Mini Mental State Examination (MMSE) score<sup>11</sup> was used as a measure of global cognitive impairment. Disease duration in AD patients was defined as the time in years between the first symptoms and the lumbar puncture. The study was approved by the Ethical Review Board of the VU University Medical Center. Patients and controls gave written informed consent to participate in the study.

### *CSF analyses*

Antisera to A $\beta$ 40 and A $\beta$ 42 were produced in rabbits by immunizing A $\beta$ 32-40 and A $\beta$ 35-42 peptides.<sup>12</sup> Antisera to A $\beta$ 38 peptide were produced in rabbits by immunization of peptide “hCys-aminohexanooyl-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-GlyOH” as described previously.<sup>12</sup> The antisera were made specific for A $\beta$ 38 by passing through the affinity column, and eluting the specific antibody at low pH. The antibody lacked reactivity against A $\beta$ 40 or A $\beta$ 42 peptides as examined by sandwich enzyme-linked immunosorbent assay and Western blot analysis (data not shown). Levels of A $\beta$ 38, A $\beta$ 40, and A $\beta$ 42 were quantified in CSF using a combination of mouse monoclonal antibody (6E10), and antibodies specific for A $\beta$ 38, A $\beta$ 40 and A $\beta$ 42 in a double antibody sandwich sandwich enzyme-linked immunosorbent assay as described<sup>13</sup>. The detection limit for the assay was 10 pg/ml for all A $\beta$  peptides. The mean of the coefficient of variation within assay was 5.0% for A $\beta$ 38, 4.6% for A $\beta$ 40, and 9.3% for A $\beta$ 42.

### *Statistical analysis*

For statistical analysis, SPSS version 11.0 was used. Mann Whitney U or Chi-square tests were used to test group and frequency differences. Spearman correlation coefficient was used for calculation of correlations. Statistical significance was set at  $p < 0.05$ . To determine the relation between A $\beta$ 38 and A $\beta$ 40 the Passing and Bablok regression method<sup>14</sup> was used, calculated by Medcalc V 4.30 Software (Medcalc Software, Mariakerke, Belgium). Applying a sensitivity of  $\geq 85\%$  or greater<sup>5</sup>, the corresponding specificities for A $\beta$ 42, and A $\beta$ 42/A $\beta$ 40 and A $\beta$ 42/A $\beta$ 38 ratios were calculated. Receiver operating curves (ROC) curves were drawn by plotting the true-positive rate (sensitivity) against the false-positive rate (100-specificity). The area under the curve (AUC) and the confidence interval of 95% (95%CI) were calculated. To assess the statistical difference of the diagnostic performance between A $\beta$ 42 and the A $\beta$ 42/A $\beta$ 40 and A $\beta$ 42/A $\beta$ 38 ratios the Hanley and McNeil method<sup>15</sup> was applied.

## Results

### *Subject characteristics and CSF levels of A $\beta$ 38, A $\beta$ 40, and A $\beta$ 42*

AD patients and controls were comparable regarding age and gender (Table). Median disease duration in AD was 4 years, ranging from 1 to 11 years. Median MMSE was 21 (range 3-28) in AD and 29 (range 25-30) in control subjects. CSF A $\beta$ 42 and the ratios of A $\beta$ 42 to A $\beta$ 40 (A $\beta$ 42/A $\beta$ 40) and A $\beta$ 42 to A $\beta$ 38 (A $\beta$ 42/A $\beta$ 38) were significantly lower in AD compared with control subjects. CSF levels of A $\beta$ 38 and A $\beta$ 40 in patients with AD and control subjects were comparable.

**Table** Subject characteristics and CSF levels of A $\beta$ 38, A $\beta$ 40, and A $\beta$ 42

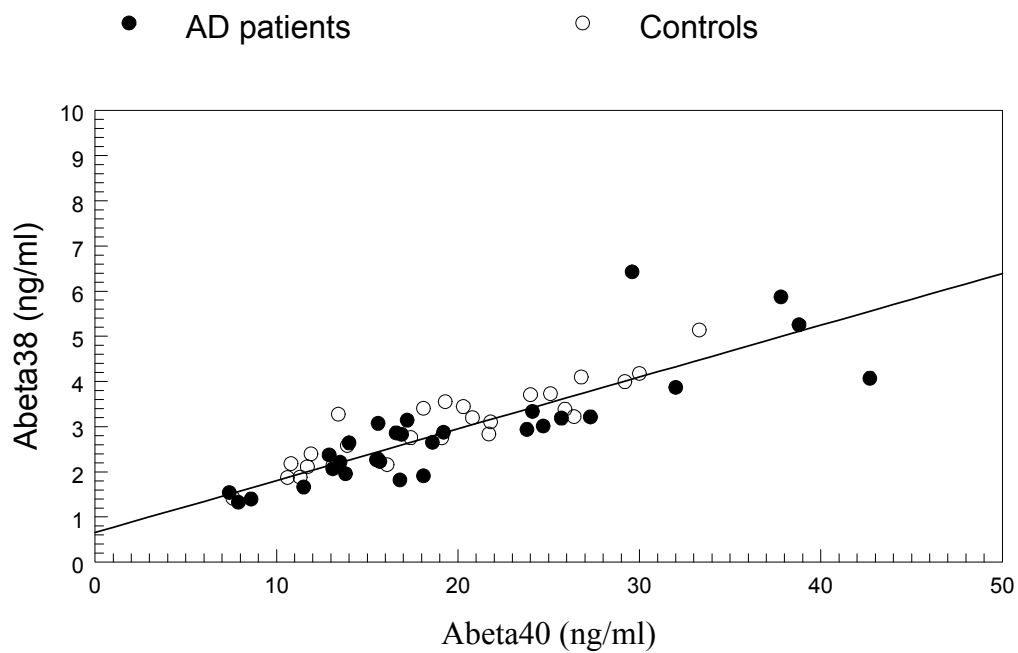
|                           | AD                  | Controls            | P-value |
|---------------------------|---------------------|---------------------|---------|
| Age                       | 60 (52-79)          | 65 (45-79)          | 0.79    |
| Sex (M/F)                 | 15/15               | 12/14               | 0.32    |
| A $\beta$ 38 (ng/mL)      | 2.7 (1.3-6.4)       | 3.2 (1.4-5.1)       | 0.22    |
| A $\beta$ 40 (ng/mL)      | 16.9 (7.4-42.7)     | 19.2 (7.6-33.3)     | 0.90    |
| A $\beta$ 42 (ng/mL)      | 0.29 (0.12-0.67)    | 0.63 (0.22-1.07)    | <0.001  |
| A $\beta$ 42/A $\beta$ 40 | 0.017 (0.006-0.048) | 0.034 (0.016-0.069) | <0.001  |
| A $\beta$ 42/A $\beta$ 38 | 0.11 (0.04-0.26)    | 0.24 (0.07-0.37)    | <0.001  |

AD = Alzheimer's disease. A $\beta$ 42/A $\beta$ 40 = ratio of A $\beta$ 42 to A $\beta$ 40; A $\beta$ 42/A $\beta$ 38 = ratio of A $\beta$ 42 to A $\beta$ 38. Values are expressed as medians (minimum-maximum).

### *Relations of CSF levels of A $\beta$ 38, A $\beta$ 40, and A $\beta$ 42*

In the whole group, a significant correlation could be shown between CSF A $\beta$ 38 and A $\beta$ 40 (R=0.89, P<0.001). The relation between A $\beta$ 38 and A $\beta$ 40 is shown in the Figure. Correlation coefficients per diagnostic group of A $\beta$ 42 versus A $\beta$ 38 are 0.46 for AD (P=0.01) and 0.58 for control subjects (P=0.02). For A $\beta$ 42 versus A $\beta$ 40 we found for AD R=0.43 (P=0.02) and R=0.65 (P<0.001) for control subjects. In the whole group, positive correlations were found between CSF A $\beta$ 38 and A $\beta$ 40 and age (R=0.30, P=0.023 and R=0.28, P=0.037, respectively). There was no

**Figure Plot of CSF A $\beta$ 38 and A $\beta$ 40 in AD and Controls**



The line represents the regression line  $Y = 0.66 + 0.115X$  correlation among CSF levels of A $\beta$ 38, A $\beta$ 40, A $\beta$ 42, and age, disease duration or MMSE in either group.

correlation among CSF levels of A $\beta$ 38, A $\beta$ 40, A $\beta$ 42, and age, disease duration or MMSE in either group.

#### *Diagnostic performance of A $\beta$ 42 and the A $\beta$ 42/A $\beta$ 40 and A $\beta$ 42/A $\beta$ 38 ratios*

Comparing AD patients with control subjects specificity for CSF A $\beta$ 42 was 88% at a sensitivity of 87% using a cut off value of 0.37 ng/mL. For the A $\beta$ 42/A $\beta$ 40 and A $\beta$ 42/A $\beta$ 38 ratios specificities were 81% and 77% at a sensitivity of 87%. Calculation of ratios did not improve the diagnostic accuracy of CSF A $\beta$ 42 (AUC A $\beta$ 42 = 0.91, 95% CI 0.80 - 0.97, AUC A $\beta$ 42/A $\beta$ 40 ratio = 0.92, 95% CI 0.81 - 0.97, and AUC A $\beta$ 42/A $\beta$ 38 ratio = 0.90, 95% CI 0.79-0.96; A $\beta$ 42 versus A $\beta$ 42/A $\beta$ 40 ratio, P= 0.88, A $\beta$ 42 versus A $\beta$ 42/A $\beta$ 38 ratio, P= 0.80).

## **Discussion**

Our data showed that CSF concentration of A $\beta$ 42 was decreased in AD, whereas CSF A $\beta$ 38 and A $\beta$ 40 levels were similar in patients with AD compared with control subjects. All three A $\beta$  peptides were related to each other, with the strongest correlation between CSF A $\beta$ 38 and A $\beta$ 40. Diagnostic accuracy of CSF A $\beta$ 42 alone was not different from the A $\beta$ 42/A $\beta$ 40 and A $\beta$ 42/A $\beta$ 38 ratios.

To our knowledge, no other study has been published, in which A $\beta$ 38, A $\beta$ 40 and A $\beta$ 42 have been estimated by a quantitative method in the same CSF samples. By employing SDS-page gel electrophoresis<sup>3</sup> it was reported that the absolute amount of CSF A $\beta$ 42 was decreased in AD, while CSF A $\beta$ 38 and A $\beta$ 40 concentrations were similar between AD and control subjects. When the amount of the single A $\beta$  peptide species was calculated relative to total A $\beta$ , an increase of CSF A $\beta$ 38 and A $\beta$ 40 was found. However, it is unclear how the total amount of A $\beta$  was measured in this study. Using the surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) technique<sup>16</sup> they found an increased peak of A $\beta$ 38 in pooled CSF from AD patients, whereas CSF A $\beta$ 40 remained unchanged. The unchanged CSF concentration of A $\beta$ 40 is consistent with previous studies.<sup>4,7,17</sup> The discrepancy of the A $\beta$ 38 findings could be attributed to the use of different methods. Furthermore, as both A $\beta$ 38 and A $\beta$ 40 are soluble peptides, they are



supposed to be released easily into the CSF, which might result in comparable concentrations in AD and controls.

The correlation between CSF A $\beta$ 42 and CSF A $\beta$ 38 or A $\beta$ 40 was most prominent in control subjects. Our findings are in line with the other study<sup>3</sup>, in which a close correlation was found among the quintet A $\beta$ 37, A $\beta$ 38, A $\beta$ 39, A $\beta$ 40 and A $\beta$ 42 in control subjects. The various C-terminally truncated A $\beta$  peptides are formed by (alternative)  $\gamma$ -secretase cleavage of APP. A $\beta$ 42 is deposited in the plaques as a result of fibrillation. In all stages of plaque formation A $\beta$ 42 is abundant, while A $\beta$ 40 and to a lesser extent A $\beta$ 38 are found in later stages of plaques maturation.<sup>18,19</sup> In some control subjects, A $\beta$ 38, A $\beta$ 40 and A $\beta$ 42 can be found in the brain, but to a much lower extent.<sup>19</sup> Analysis of CSF rests upon the assumption that CSF reflects the biochemical processes taking place in the brain. The decrease of A $\beta$ 42 in CSF thus can be explained by deposition of this peptide in the brain of patients with AD. Our findings further support this hypothesis, since we found only A $\beta$ 42 to be decreased in CSF, which correlated poorly to A $\beta$ 38 and A $\beta$ 40 in AD.

We found no difference in diagnostic accuracy of CSF A $\beta$ 42 compared to the A $\beta$ 42/A $\beta$ 40 and A $\beta$ 42/A $\beta$ 38 ratios for the differentiation of AD from control subjects. The A $\beta$ 42/A $\beta$ 40 and A $\beta$ 42/A $\beta$ 38 ratios are considered to give information about the disease progression, typically in the early stage of disease, as the cerebral deposition of A $\beta$ 42 probably starts already before the disease becomes clinically overt.<sup>20</sup> This is in line with an earlier report showing an increased ratio of A $\beta$ 40/A $\beta$ 42 before the clinical onset of AD.<sup>17</sup> CSF A $\beta$ 42 alone is considered to be a stage marker, reflecting the presence of the disease at a certain stage.<sup>20</sup> It would be of interest to investigate the ratio of A $\beta$ 42 to A $\beta$ 40 and A $\beta$ 38 in a group of patients with mild cognitive impairment, observed longitudinally, to be informed when A $\beta$ 42 starts to decrease in CSF as compared to A $\beta$ 38 and A $\beta$ 40, in relation with clinical progression.

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## **CHAPTER 5**

### **CSF A $\beta$ 42, TAU AND PHOSPHORYLATED TAU IN ALZHEIMER'S DISEASE VERSUS FRONTOTEMPORAL DEMENTIA**



## **CHAPTER 5.1**

### **Amyloid $\beta$ 42 (1-42) and phosphorylated tau in CSF as markers for early onset Alzheimer's disease**

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## **Abstract**

*Objective:* To determine the diagnostic value of CSF amyloid  $\beta$  1-42 (A $\beta$ 42), CSF total tau and CSF tau phosphorylated at threonine 181 (Ptau-181) in early onset AD (EAD) vs frontotemporal lobar degeneration (FTLD).

*Methods:* Levels of A $\beta$ 42, total tau and Ptau-181 in CSF were measured using commercially available ELISAs in 47 EAD patients, 28 FTLD patients and 21 non-demented controls.

*Results:* CSF A $\beta$ 42 was significantly lower, and CSF total tau and CSF Ptau-181 significantly higher in EAD patients compared to FTLD patients and controls. There was an increase in diagnostic accuracy for CSF Ptau-181 vs CSF total tau ( $P=0.067$ ). Combining low CSF A $\beta$ 42 and high CSF Ptau-181 allowed EAD patients to be separated from FTLD patients with a sensitivity of 72% and a specificity of 93%. Logistic regression analysis with CSF A $\beta$ 42 and CSF Ptau-181 as independent continuous variables resulted in correct classification of 46/47 (98%) EAD patients and 23/28 (82%) FTLD patients. The diagnostic accuracy for EAD was independent of gender, disease duration or disease severity.

*Conclusion:* The combination of CSF A $\beta$ 42 and CSF Ptau-181 may help differentiating EAD from FTLD.

## Introduction

In early onset AD (EAD), disorders of language, praxis or executive functions are more common than memory problems.<sup>1,2</sup> Compared to the other major types of early onset dementia<sup>2</sup> EAD is most difficult to distinguish from frontotemporal lobar degeneration (FTLD) on clinical and radiological grounds, especially in the early phase of disease. The clinical and radiological overlap calls for more specific diagnostic tools.

The combination of decreased CSF levels of amyloid  $\beta$  1-42 (A $\beta$ 42) and increased levels of total tau has been found to be a highly sensitive discriminator of AD from normal aging.<sup>3,4</sup> In FTLD, levels of CSF A $\beta$ 42 have been found to be normal or moderately low<sup>5</sup>, while CSF total tau was either normal<sup>6</sup> or increased.<sup>7-9</sup> The few studies that investigated CSF phosphorylated tau<sup>10-12</sup> all showed better separation between AD and FTLD compared to total tau. However, in two studies patients were not matched for age<sup>10,11</sup>, corrected for by analysis of variance with age as co-variate<sup>10</sup>. A third study included an older AD and FTLD population of comparable age<sup>12</sup>, but the prevalence of AD in late onset dementia is considerably higher than FTLD. When *a priori* chances of dementia syndromes differ according to age, the usefulness of biomarkers to distinguish them becomes less relevant. A recent study<sup>13</sup> demonstrated that the prevalence of AD and FTLD may be comparable in an early onset dementia population. To date, only one study has described the value of two biomarkers -neurofilament protein and total tau- for the differential diagnosis of EAD and FTLD.<sup>14</sup> We investigate the diagnostic value of the currently most specific commercially available biomarkers CSF A $\beta$ 42, CSF total tau and CSF Ptau-181 in a large group of EAD patients, FTLD patients and non-demented age-matched controls.

## Materials and methods

### *Subjects*

Between October 2000 and June 2003 47 EAD patients and 22 FTLD patients (12 with FTD, seven SD and three PA) were consecutively investigated at the Alzheimer Center of the VU University Medical Center (VUMC), Amsterdam. An additional six FTLD patients were recruited from the Erasmus Medical Center, Rotterdam (four FTLD and two SD). In all EAD and FTLD patients symptoms started before the age of 65. All patients underwent a standardized investigative battery, including medical history, physical and neurological examination, screening



laboratory tests, psychometric evaluation, EEG, and brain MRI or CT. In two EAD and five FTLD patients with normal or inconclusive findings on structural imaging, Single Photon Emission Computed Tomography (SPECT) with 99mTc-hexamethyl propyleneamine oxime was performed. Dementia severity was assessed by the clinical dementia rating scale (CDR, all EAD and FTLD patients)<sup>15</sup> and Mini Mental State Examination (MMSE) score (all EAD patients and controls, 21 out of 28 FTLD patients).<sup>16</sup> Disease duration was defined as the time in years between the first symptoms (by history) and the first clinical diagnosis. At that time all patients underwent a lumbar puncture. The initial diagnoses were made in conference by a team of neurologists, neuropsychologists, a neurophysiologist, a psychiatrist and a radiologist. Diagnosis of probable AD was made by exclusion according to the NINCDS-ADRDA criteria<sup>17</sup>; for FTLD we used the clinical diagnostic criteria of Neary et al.<sup>18</sup>

The team involved in the diagnostic work-up was blinded to the results of the CSF analyses. For most of the EAD and FTLD patients, clinical diagnoses were revisited and confirmed after a minimum follow up period of six months and used in the analysis as the golden standard. The diagnoses of the six FTLD cases from the Erasmus Medical Center were confirmed after a follow up period of at least one year. In the 22 FTLD cases from the VUMC diagnoses were revisited and confirmed by an independent neurologist. The three EAD patients, from whom CSF was collected in June 2003 had been referred for a second opinion, and had been extensively evaluated elsewhere. They underwent the same diagnostic procedure as all the other patients. In 19 EAD patients disease started with memory problems, while in the rest of the patients, aphasia, apraxia or executive problems were initial features. Two EAD patients had first-degree family members with confirmed AD starting before the age of 65, while the other EAD patients with a positive family history for dementia had family members with late-onset type of AD. No further genetic analysis took place in these patients. There were two FTLD patients with a positive family history for early onset dementia; in one FTLD patient the P301L tau mutation was present. The control group consisted of 21 non-demented subjects with a maximum age of 70. The group included 13 subjects with subjective memory complaints, who were seen at the Alzheimer Center of the VU University Medical Center and had undergone the same examinations as the patients; five healthy spouses of patients with no memory complaints; two subjects with a positive family history for AD and one patient with intracranial hypertension. None of the control subjects had developed dementia after a follow up period of at least 6 months. The study was approved by the

ethical review boards of both the VU and Erasmus Medical Centers. All patients and controls gave written informed consent to participate in the study.

### *CSF analysis*

CSF was obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space, and 12 ml was collected in polypropylene tubes. At the same time a serum sample was taken. Within an hour, CSF and serum samples were centrifuged at 3000 rpm for 10 minutes at 4°C. A small amount of CSF was used for routine analysis, including total cells, total protein, and erythrocytes. CSF was aliquoted in polypropylene tubes of 0.5 or 1 ml, and stored at -80°C until analysis. CSF A $\beta$ 42, CSF total tau and CSF Ptau-181 were measured by commercially available sandwich ELISAs (Innotest  $\beta$ -amyloid (1-42)<sup>19</sup>, Innotest hTAU-Ag<sup>20</sup> and Innotest Phosphotau (181P); Innogenetics, Ghent, Belgium).<sup>21</sup> All CSF analyses were performed at the department of Clinical Chemistry of the VUMC.

### *Statistical analysis*

For statistical analysis, SPSS version 11.0 was used. In the study population none of the variables, except age, were normally distributed. Non-parametric analyses (Kruskall Wallis followed by the Mann Whitney U test) were used to compare medians of age, disease duration, disease severity (CDR and MMSE), CSF A $\beta$ 42, CSF total tau and CSF Ptau-181. Statistical significance was set at  $p < 0.05$ . To calculate correlations between sex, age, disease duration, disease severity and CSF A $\beta$ 42, CSF total tau and CSF Ptau-181 Spearman correlation coefficient was used. Receiver Operating Characteristic (ROC) curves were drawn by plotting the true-positive rate (sensitivity) against the false-positive rate (100-specificity). Based on the assumption that the clinical criteria for AD provide a sensitivity of approximately 85%<sup>22</sup>, we applied a sensitivity of  $\geq 85\%$  for each individual biomarker in accordance with the Reagan Consensus report.<sup>23</sup> We calculated the corresponding specificities and cut-off values, using EAD as positive cases and FTLN or controls as negative cases. The areas under the ROC curves (AUC) and standard errors (SE) were calculated by Medcalc V 4.30 Software (Medcalc Software, Mariakerke, Belgium). To assess the statistical difference of the discriminating power of CSF total tau and CSF Ptau-181 as well as CSF A $\beta$ 42 and CSF Ptau-181 from ROC curves the Hanley

and McNeil method was applied using the same statistical program.<sup>24</sup> To differentiate EAD and FTLD a logistic regression analysis with backward stepwise selection method was used as a statistical modeling technique to estimate the simultaneous impact of the variables sex, disease duration, disease severity (CDR), CSF A $\beta$ 42, and CSF Ptau-181.

## Results

EAD patients were well matched for age with FTLD patients (Table 1). Furthermore, age at onset of disease was comparable between the two patient groups. In EAD and control subjects women were overrepresented, while in FTLD there was a surplus of men. There was a tendency towards a longer disease duration in EAD patients compared to FTLD patients. EAD patients were more functionally impaired than FTLD patients as measured by the CDR and MMSE.

CSF A $\beta$ 42 was significantly lower, and CSF total tau and Ptau-181 were significantly higher in EAD patients than FTLD patients and control subjects. Total tau was increased in FTLD patients compared with control subjects ( $P=0.03$ ), while no difference in CSF A $\beta$ 42 and CSF Ptau-181 could be demonstrated ( $P=0.2$  and  $P=0.7$ ). There was a trend of increased CSF Ptau-181 in patients with the temporal variant of FTLD ( $P=0.06$ ).

No significant associations were found between the three biomarkers and gender, disease duration or disease severity in EAD, FTLD and controls. In FTLD, a negative correlation between CSF A $\beta$ 42 and age could be demonstrated ( $r=-0.39$ ,  $P=0.04$ ). Furthermore, there was a trend towards a positive correlation between age and CSF Ptau-181 in control subjects ( $r=0.39$ ,  $P=0.08$ ). Positive correlations were found between CSF total tau and CSF Ptau-181 in each group (EAD:  $r=0.95$ ,  $P<0.001$ ; FTLD:  $r=0.8$ ,  $P<0.001$ ; controls:  $r=0.85$ ,  $P<0.001$ )

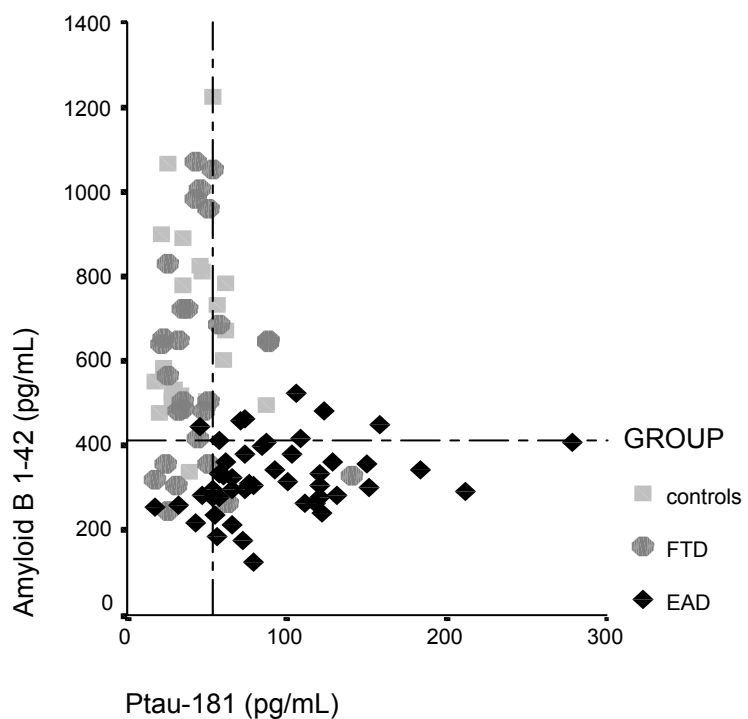
Sensitivity and specificity for each biomarker were calculated using ROC analyses. Using a cut off value of 413 pg/mL for CSF A $\beta$ 42, EAD patients could be separated from FTLD patients with a sensitivity of 85% and a specificity of 75%. Sensitivity and specificity values for CSF total tau were 85% and 74% at a cut off value of 377 pg/mL. For CSF Ptau-181 sensitivity was 85% and specificity 82% at a cut off value of 54 pg /mL. There was a tendency towards a significant increase in diagnostic accuracy for CSF Ptau-181 compared with CSF total tau (CSF total tau: AUC = 0.813, 95% CI 0.706-0.894; CSF Ptau-181: AUC = 0.866, 95% CI 0.767-0.933  $P=0.067$ ).

**Table 1: Demographic data and CSF analyses per diagnostic category**

|                      | EAD (n=47)    | FTLD (n=28)    | Controls (n=21) | P-value* | P-value** |
|----------------------|---------------|----------------|-----------------|----------|-----------|
| Age (yrs)            | 59 (52-68)    | 60 (43-68)     | 62 (39-70)      | 0.7      | 0.9       |
| Sex (F:M)            | 29:18         | 10 :18         | 14 :7           | 0.03     | 0.7       |
| Disease duration     | 4 (1-11)      | 3 (1-11)       | --              | 0.08     | --        |
| CDR                  | 1 (1-3)       | 1 (0.5-2)      | --              | 0.002    | --        |
| MMSE                 | 20 (3-28)     | 25 (3-29)      | 29 (27-30)      | 0.01     | < 0.001   |
| A $\beta$ 42 (pg/ml) | 307 (124-525) | 603 (245-1072) | 604 (337-1224)  | < 0.001  | < 0.001   |
| Total tau (pg/ml)    | 642 (75-2692) | 330 (65-1527)  | 191 (95-587)    | <0.001   | <0.001    |
| Ptau-181 (pg/ml)     | 79 (18-279)   | 41 (18-141)    | 35 (18-87)      | < 0.001  | < 0.001   |

EAD = early onset Alzheimer's disease; FTLD = Frontotemporal Lobar Degeneration; CDR = Clinical Dementia Rating; MMSE = Mini Mental State Examination; M = male, F = female; yrs = years; Ptau-181 = tau protein phosphorylated at threonine 181; A $\beta$ 42 = amyloid  $\beta$  1–42. Disease duration is defined as the time in years between the first symptoms (by history) and the first clinical diagnosis. Values are expressed as medians (minimum-maximum). P-values refer to statistical difference between EAD vs FTLD (\*) and EAD vs controls (\*\*).

**Figure 1** Plot CSF amyloid  $\beta$  1-42 and CSF tau phosphorylated at threonine 181 in EAD vs FTLD



EAD=early onset Alzheimer's disease, FTLD= frontotemporal lobar degeneration. Ptau-181 = tau phosphorylated at threonine 181. Dotted lines represent the cut-off values for EAD vs FTLD

**Table 2** Cross tabulation of CSF amyloid  $\beta$  1-42 and CSF tau phosphorylated at threonine 181 in EAD, FTLD and controls

|   | EAD<br>(N=47) | FTLD<br>(N=28) | Controls<br>(N=21) |
|---|---------------|----------------|--------------------|
| Two markers positive<br>A $\beta$ 42 $\leq$ 413 pg/mL and Ptau-181 $>$ 54 pg/mL                 | 34            | 2              | 0                  |
| One marker positive: A $\beta$ 42<br>A $\beta$ 42 $\leq$ 413 pg/mL and Ptau-181 $\leq$ 54 pg/mL | 6             | 5              | 1                  |
| One marker positive: Ptau-181<br>Ptau-181 $>$ 54 pg/mL and A $\beta$ 42 $>$ 413 pg/mL           | 6             | 3              | 5                  |
| Two markers negative<br>A $\beta$ 42 $>$ 413 pg/mL and Ptau-181 $\leq$ 54 pg/mL                 | 1             | 18             | 15                 |

EAD = early onset Alzheimer's disease; FTLD = Frontotemporal Lobar Degeneration.

Ptau-181 = tau protein phosphorylated at threonine 181; A $\beta$ 42 = amyloid  $\beta$  1-42

No difference was present in diagnostic accuracy for CSF A $\beta$ 42 and CSF Ptau-181 as demonstrated by the AUC values (CSF A $\beta$ 42: AUC = 0.860, 95% CI 0.760-0.929; CSF Ptau-181: AUC = 0.866, 95% CI 0.767-0.933; P=0.92).

In EAD versus control subjects sensitivity for CSF A $\beta$ 42 was 96% at a specificity of 95% using a cut off value of 471 pg/mL. Specificity for CSF total tau was 90% at a sensitivity of 85% with a cut off value of 369 pg/mL. For CSF Ptau-181 sensitivity was 85% and specificity 76% at a cut off value of 54 pg/mL. Diagnostic accuracy of CSF A $\beta$ 42 was better than CSF Ptau-181 (CSF A $\beta$ 42: AUC = 0.981, 95% CI 0.918-0.998; CSF Ptau-181: AUC = 0.879, 95% CI 0.783-0.943; P=0.01). No difference was present in diagnostic accuracy of CSF total tau versus CSF Ptau-181 (P=0.27).

In Table 2 the number of EAD patients, FTLD patients and control subjects are depicted with at least one marker being positive or negative, or with the combination of CSF A $\beta$ 42 and CSF Ptau-181 being positive or negative, using abovementioned cut off values for EAD vs FTLD. These findings are visualised in Figure 1. For EAD vs FTLD, sensitivity and specificity for the combination of A $\beta$ 42 and Ptau in the AD range is 72% and 93%. For EAD vs controls, the

combination of A $\beta$ 42 and total tau gives a sensitivity of 81% and a specificity of 100% using the cut off values for EAD vs controls.

Positive and negative likelihood ratios (+LR and -LR) for the differentiation between EAD vs FTLD are calculated using the data depicted in table 2. The combination of two markers being positive compared with at least one marker negative gives a +LR of 10 and a -LR of 0.30. A negative value of both biomarkers compared with at least one positive gives a +LR of 2.72 and a -LR of 0.03.

When using parametric statistics (univariate analysis of variance) after log-transformation of the variables no influence of the covariates MMSE and disease duration on the primary variables A $\beta$ 42, total tau and Ptau-181 could be demonstrated. Logistic regression analysis with diagnosis (EAD or FTLD) as dependent variable and CDR, CSF A $\beta$ 42 and Ptau-181 as independent variables resulted in correct classification of 44/47 (94%) EAD patients and 24/28 (86%) FTLD patients. Removing CDR from the model correctly classified 46/47 (98%) EAD patients and 23/28 (82%) FTLD patients. Disease duration or gender did not affect the outcome of the model.

## Discussion

In this study, we found a high diagnostic accuracy for the combination of low CSF A $\beta$ 42 and high CSF Ptau-181 in differentiating EAD from FTLD. The diagnostic accuracy for EAD achieved by this combination of markers was independent of gender, disease duration and disease severity. Furthermore, we confirmed the results of other studies describing an increase in diagnostic accuracy for CSF Ptau-181 compared with CSF total tau in differentiating AD from FTLD.

Our observation of decreased CSF levels of A $\beta$ 42 in EAD patients is consistent with two previous studies in which *early onset* AD and sporadic (or *late onset*) AD (LAD) were compared.<sup>6,25</sup> Although levels of CSF A $\beta$ 42 in EAD and LAD were comparable in one of these studies<sup>6</sup>, the significantly lower CSF A $\beta$ 42 values in EAD compared with LAD in the second study<sup>25</sup> are suggestive of pathophysiological heterogeneity. It could be argued that EAD is a more ‘pure’ form of AD, in which the formation of neuritic plaques by overproduction of A $\beta$ 42 is a relatively early and prominent phenomenon, whereas in LAD multiple (vascular) pathogenic factors are thought to play a role.<sup>26</sup> The heterogeneity of AD could also explain the discordance of CSF

markers between different studies<sup>27</sup>, and support the notion of separating EAD from LAD patients in research on diagnostic markers.

Our findings of an increase of CSF Ptau-181 in five of the 28 FTLD patients do not correspond with those from two previous studies<sup>10,21</sup>, in which Ptau-181 was found to be significantly lower in FTLD patients compared to AD patients and controls. A possible explanation for this discrepancy could be that the authors compared younger FTLD patients (mean age of 65 years) to older AD patients (mean age of 74 years) and controls (mean age of 72 years). In our study we found a trend towards a positive correlation between age and Ptau-181 in controls, which might have been stronger if subjects with a broader age range had been included. An age-related increase of CSF total tau in healthy individuals has previously been demonstrated<sup>28,29</sup>, and may reflect age related neuronal or axonal degeneration. Recently, a positive correlation between CSF Ptau-231 and age was found in a population of depressed subjects.<sup>30</sup> These consistent correlations between age and total tau and Ptau stress the importance of comparing only groups that are strictly matched for age.

Another explanation for the discrepant results between our study and the aforementioned studies<sup>10,21</sup> could be the heterogeneity in underlying pathology of FTLD.<sup>31</sup> Although the extent of abnormal phosphorylation of tau is supposed to be higher in tau deposits in the brain of AD patients compared with FTLD patients, phosphorylation of tau at threonine 181 itself is a normal phenomenon<sup>32</sup>, which could either be decreased<sup>10</sup> or increased, as in some of our FTLD patients. Two of the FTLD patients had an increase of CSF total tau without an increase of CSF Ptau-181 resulting in a better diagnostic accuracy of CSF Ptau-181. Overall, the increase of CSF total tau in a subset of FTLD patients could be a reflection of tau deposits, which are found in a minority of FTLD patients.<sup>31</sup> However, the hypothesis of a direct association between tau pathology and an increase of CSF total tau remains unproven as demonstrated recently by normal CSF total tau levels in FTLD patients with tau mutations that cause intracerebral tau-deposition.<sup>33</sup>

It could be argued that our results lack biological validity in the absence of post-mortem verification. This applies to many of the studies published in the field of diagnostic markers and in our opinion does not invalidate the results. We tried to achieve the highest diagnostic certainty by means of the same rigorous diagnostic work up in every patient, use of all available imaging information, including a high resolution MRI protocol in all patients, and ensuring clinical follow



up of at least 6 months. From class I studies (prospective studies with neuropathological confirmation) it is known that the NINCDS-ADRDA for probable AD have a high sensitivity but a moderately high specificity.<sup>22,34</sup> Although none of the other diagnostic measurements (neuropsychological tests, MRI scan) have neuropathological validation, serial tests should increase the overall specificity of the diagnosis.<sup>34</sup>

The difference in diagnostic value of the single biomarkers for EAD and FTLD on the one hand and EAD and controls on the other hand is noteworthy. For the differentiation of EAD from FTLD, CSF Ptau-181 seems to be a slightly more specific marker than CSF A $\beta$ 42; seven out of 28 FTLD patients had a CSF level of A $\beta$ 42 below the cut off value. Although plaques are not a common feature in the sporadic form of FTLD, several authors found decreased CSF levels of A $\beta$ 42 in a subset of FTLD patients<sup>5</sup>, which may be related to the presence of an Apo E  $\epsilon$ 4 allele or older age<sup>35</sup> as in our patients. For the differentiation of EAD from age-matched elderly controls CSF A $\beta$ 42 alone is found to be the most sensitive and specific marker, followed by CSF total tau, whereas CSF Ptau-181 was increased in five out of 21 controls. This increase of CSF Ptau-181 could be related to aging, but there remains the possibility that these five subjects may develop AD in the future, particularly as four of the five had subjective memory complaints. This hypothesis is supported by a recent study showing that high CSF levels of Ptau-231 at baseline, but not total tau levels, correlated with cognitive decline and conversion from mild cognitive impairment (MCI) to AD.<sup>36</sup>

According to the Reagan Consensus Report<sup>23</sup> an ideal biomarker needs to have a specificity of 75-85% and a sensitivity of  $\geq 85\%$  to be clinically useful. To date, no single marker has been found to be specific enough to differentiate AD from other dementias, and the use of a combination of markers has been advocated to increase specificity.<sup>37</sup> Indeed, in our study only two FTLD patients (both with PA) had a value of both markers in the AD range (specificity 93%), an increased specificity of more than 10% compared with the use of CSF A $\beta$ 42 or CSF Ptau-181 alone. It would be informative to collect further autopsy data from these two PA patients. Although the majority of PA cases presented in the literature have non-AD pathology, several case reports of patients with non-fluent aphasic syndromes have been described, who revealed typical AD pathological features at autopsy.<sup>2</sup>

For the use of the markers to assist with the differential diagnosis of EAD and FTLD in clinical practice, a low negative likelihood ratio may be even more important.<sup>23</sup> A test to exclude EAD will be of great benefit to the patient and would provide the clinician the opportunity to direct the diagnostic effort elsewhere. Furthermore, it has been demonstrated by retrospective neuropathological studies that the specificity of the NINCDS-ADRDA criteria for the differential diagnosis of AD vs FTLD is only 23%.<sup>38</sup> A negative laboratory test may be more informative to the clinician regarding the neuropathological nature of the underlying type of dementia, when clinical and radiological criteria fail to do so. Our findings of a very low -LR (0.03) when both markers are negative make this combination of markers quite robust.

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## **CHAPTER 5.2**

**CSF tau and Abeta42 are not useful in the diagnosis of frontotemporal lobar degeneration**

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Frontotemporal lobar degeneration (FTLD),<sup>1</sup> a neurodegenerative disorder presenting with a spectrum of behavior changes, executive disturbances, or aphasia, is often unrecognized. Patients with FTLD are many times considered to have a psychiatric disorder or Alzheimer disease (AD). Imaging studies and psychometric testing can be normal at an early stage.<sup>2</sup> CSF biomarkers have been considered in the diagnosis of FTLD. One study found that the combination of CSF tau and amyloid  $\beta$  (1–42) (A $\beta$ 42) provided assistance in the diagnosis of FTLD.<sup>4</sup> We investigated CSF tau and A $\beta$ 42 in FTLD compared with age-matched AD patients and cognitively healthy control subjects.

*Methods* Thirty-five patients with FTLD (18 frontotemporal dementia, 11 semantic dementia, and 6 progressive nonfluent aphasia) were compared with 51 patients with probable AD<sup>3</sup> and 27 nondemented control subjects. All patients underwent a standard medical history, physical and neurologic examination, screening laboratory tests, psychometric tests, EEG, MRI, or CT. 99mTc-Hexamethylpropyleneamine oxide SPECT was performed in seven cases with normal or inconclusive findings on structural neuroimaging. Only patients whose clinical diagnoses were evaluated by a multidisciplinary team and supported by either structural or functional neuroimaging were included. All but two of the FTLD cases were sporadic. Genetic screening took place in one of the familial cases, yielding a P301L mutation. The clinical diagnosis was confirmed pathologically in one case, showing neuronal degeneration without tau pathology. The Clinical Dementia Rating Scale (CDR)<sup>5</sup> was used to assess dementia severity.

The control group consisted of 16 subjects with subjective memory complaints, who underwent the same examinations as the patients, 5 cognitively healthy subjects with a positive family history, as well as 6 healthy spouses of patients with no memory complaints.

CSF was obtained by lumbar puncture between the L3 to L4 or L4 to L5 intervertebral space after informed consent. Within an hour, CSF samples were centrifuged at 3,000 rpm for 10 minutes at 4 °C followed by storage in polypropylene tubes at –80 °C until analysis. CSF tau and A $\beta$ 42 were determined by sandwich ELISA (Innotest  $\beta$ -amyloid(1–42) and Innotest hTAU-Ag; Innogenetics, Ghent, Belgium).

*Results* Clinical, demographic, as well as CSF tau and A $\beta$ 42 data are displayed in the table. No significant differences in CSF tau between the FTLD subgroups were found. In progressive

nonfluent aphasia, A $\beta$ 42 was lower than in frontotemporal dementia ( $p = 0.033$ ) and semantic dementia ( $p = 0.015$ ).

A cut-off value of 908 pg/mL for CSF tau distinguished FTLD from AD at a sensitivity of 86% and a specificity of 26%. To distinguish FTLD from controls, a CSF tau cut-off of 193 pg/mL yielded a sensitivity of 86% at a specificity of 41%. For CSF A $\beta$ 42, a cut-off value of 315 pg/mL distinguished FTLD from AD at a sensitivity of 86% and a specificity of 59%. The number of FTLD patients that was correctly classified by a CSF tau range between 193 and 908 pg/mL and a CSF A $\beta$ 42 value higher than 315 pg/mL was 21 (60%).

*Discussion* Even though significant differences were found, there was extensive overlap of CSF tau and A $\beta$ 42 values between FTLD, AD, and control subjects. In a relatively young population where the a priori chance of FTLD is at best 50%,<sup>6</sup> the positive predictive value of a CSF tau value between 193 and 908 pg/mL combined with a CSF A $\beta$ 42 value higher than 315 pg/mL would be 51% with a negative predictive value of 52%, which is not above chance level.

Therefore, we conclude that measurement of tau and A $\beta$ 42 in CSF is not useful for the diagnosis of FTLD.

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**Table Clinical and demographic data and values of CSF biomarkers**

| Data                    | FTLD, N = 35    | AD, N = 51      | Controls, N = 27 | Statistical significance                            |
|-------------------------|-----------------|-----------------|------------------|---|
| Age, y                  | 61 (51–85)      | 63 (53–78)      | 66 (39–76)       | P = 0.50  |
| Sex, M:F                | 23:12           | 22:29           | 11:16            | P < 0.001   |
| CDR                     | 1 (0.5–2)       | 1 (1–3)         | —                | P = 0.009   |
| Disease duration, y     | 3 (1–11)        | 4 (1–11)        | —                | P = 0.014   |
| CSF tau, pg/mL          | 353 (49–1,740)  | 632 (195–1,822) | 298 (95–993)     | FTLD vs AD, P = 0.002<br>FTLD vs controls, P = 0.03 |
| CSF A $\beta$ 42, pg/mL | 565 (178–1,225) | 298 (124–592)   | 634 (232–1,243)  | FTLD vs AD, P < 0.001<br>FTLD vs controls, P = 0.13 |

Values are medians (range). For statistical analysis, nonparametric tests were used (Kruskal–Wallis, Mann–Whitney); differences in male/female distribution were analyzed using the  $\chi^2$  test. FTLD = frontotemporal lobar degeneration; AD = Alzheimer disease; CDR = Clinical Dementia Rating Scale; A $\beta$ 42 = amyloid  $\beta$  (1–42), y = years

## **CHAPTER 6**

### **BIOMARKER PROFILES AND THEIR RELATION TO CLINICAL VARIABLES IN MILD COGNITIVE IMPAIRMENT**

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## **Abstract**

The aim of the study was to compare clinical variables between MCI patients at different risk for Alzheimer's disease (AD) according to their biomarker profile. Fifty-four % out of 39 MCI patients had a low A $\beta$ 42 and high tau in cerebrospinal fluid (CSF) (high-risk), 26% either a low CSF A $\beta$ 42 or high CSF tau (intermediate-risk) and 20% a normal CSF A $\beta$ 42 and tau (low-risk). Both high- and intermediate-risk subjects differed from the low-risk group in episodic memory, executive functions and the preclinical AD scale (PAS), which combines a set of clinical parameters. Subjects at high-risk did not differ from subjects with an intermediate-risk. A $\beta$ 42 levels correlated with the MTA and PAS scores, tau levels with episodic memory. These correlations suggest that the biomarkers are not independent when compared to the other AD markers. Longitudinal studies are necessary to interpret the correlations between biomarkers, imaging, and neuropsychological markers.

## Introduction

Mild cognitive impairment (MCI) is considered to be a transitional state between normal aging and dementia<sup>16</sup> and 10-15% of the patients with MCI progress to Alzheimer type dementia (AD) each year.<sup>17</sup> Several studies have shown that a subgroup of MCI patients has low cerebrospinal fluid (CSF) amyloid  $\beta$  1-42 (A $\beta$ 42) and high total tau (tau) levels that are considered indicative for AD. However, most of these studies have been performed in specific research settings.<sup>1,2,20</sup> Little is known about how frequently such a typical AD biomarker profile is seen in MCI patients selected from a setting that reflects daily clinical practice.<sup>32</sup>

No cross-sectional studies investigated to what extent MCI patients with an AD biomarker profile differ from MCI patients with a normal biomarker profile with respect to other clinical markers or risk factors of AD. This is important to know for two reasons. First, investigating the relation between biomarkers and other markers of AD will show whether these markers are independent predictors for AD at baseline. Second, it will yield information regarding the pathophysiology of AD in the early stage.

The aim of the present study is threefold. First, we investigate how many subjects with amnesic MCI from our Memory Clinic display high-risk (low CSF A $\beta$ 42 and high CSF tau), intermediate risk (low CSF A $\beta$ 42 and normal CSF tau *or* normal CSF A $\beta$ 42 and high CSF tau), and low-risk (normal CSF A $\beta$ 42 and normal CSF tau) biomarker profiles. Secondly, we compare age, disease duration, MMSE, Apo E genotype, neuropsychological test performance, and medial temporal lobe atrophy (MTA) score among these groups. In addition, we compare the score on the preclinical Alzheimer's disease Scale (PAS)<sup>29</sup> between the groups with different biomarker risk profiles. The PAS is a scale that applies a set of clinical parameters to estimate the risk for AD in MCI patients. Finally, we investigate the correlation between the two biomarkers and the other risk factors for AD.

## Patients and methods

### *Patients*

Thirty-nine patients younger than 85 years with amnesic MCI were consecutively recruited at the Alzheimer Center of the VU University Medical Centre (VUMC), Amsterdam, between January 2001 and November 2003. Patients were referred to our memory clinic by general practitioners (N=30) or by other neurologists/specialists as second opinions (N=9). Diagnosis of amnesic MCI



was made according to the criteria of Petersen et al.<sup>17</sup>, indicating that a patient had subjective and objective memory impairment with no interference with daily activities, and no dysfunction in other cognitive domains. The patients underwent a standardized clinical assessment, including medical history, physical and neurological examination, laboratory tests, psychometric evaluation, EEG, and brain MRI or CT. The initial diagnoses were made in conference by a team of neurologists, neuropsychologists, a neurophysiologist, a psychiatrist, and a radiologist. The team involved in the diagnostic work-up was blinded to the results of the CSF analyses. The Mini Mental State Examination (MMSE) score<sup>7</sup> was used as a measure of global cognitive impairment. Disease duration was defined as the time in years between the first symptoms (by history) and the lumbar puncture (LP) for CSF sampling. Median time between first assessment and LP was 2 months (range 0-23). All patients met criteria for amnesic MCI at the time of the LP. The ethical review board of the VUMC approved the study and all subjects gave written informed consent.

#### *CSF analysis*

CSF was obtained by lumbar puncture (LP) between the L3/L4 or L4/L5 intervertebral space and collected in polypropylene tubes. Within an hour, CSF samples were centrifuged at 3000 rpm for 10 minutes at 4°C. A small amount of CSF was used for routine analysis, including total cells, total protein, and erythrocytes. CSF was aliquoted in polypropylene tubes of 0.5 or 1 ml, and stored at -80°C until analysis. CSF A $\beta$ 42 and tau were measured by commercially available sandwich ELISAs (Innotest  $\beta$ -amyloid (1-42)<sup>27</sup> and Innotest hTAU-Ag<sup>26</sup>, Innogenetics, Ghent, Belgium). All CSF analyses were performed at the department of Clinical Chemistry of the VUMC.

#### *Calculation of the cut off values*

The optimal cut-off values for CSF A $\beta$ 42 and tau were set at data obtained from 92 probable AD patients<sup>15</sup> and 38 controls also recruited at the Alzheimer Centre, VUMC. After drawing Receiver Operating Characteristics (ROC) curves, we applied a sensitivity of  $\geq 85\%$  for each individual biomarker in accordance with the Ronald and Nancy Reagan Consensus report.<sup>25</sup> The corresponding specificities and cut-off values were calculated. Sensitivity and specificity values for CSF A $\beta$ 42 were 86% and 89% using a cut off level of  $\leq 494$  pg/mL. For CSF tau sensitivity and specificity were 89% and 74% at a cut off level of  $> 356$  pg/mL.

### *Apo E genotype*

DNA was isolated from 10 ml EDTA blood and was available from 36 out of 39 patients. ApoE genotype was determined with the Light Cycler ApoE mutation detection method (Roche diagnostics GmbH, Mannheim, Germany). Patients were dichotomized on the basis of no or  $\geq 1$   $\epsilon 4$  allele.

### *MRI analysis*

MRI scans were made on a 1.0 (N=30) or 1.5 (N=4) Tesla scanner (Siemens), in coronal (mp-rage, slice thickness 1.5 mm) and axial direction (FLAIR, slice thickness 5 mm). In 3 MCI patients no MRI was made because of claustrophobia (N=1) or the presence of a pacemaker (N=2). Two other patients were also excluded from MRI analysis, one patient because the time between LP and MRI scan was  $> 12$  months and in another patient because no coronal scan was available. Median time between LP and MRI scan was 2 months (range 0-10). The MTA score was rated visually according to the method of Scheltens et al.<sup>30</sup> The MTA score is based on a visual estimation of the volume of the medial temporal lobe, including the hippocampus proper, dentate gyrus, subiculum, and parahippocampal gyrus, and the volume of the surrounding CSF spaces, in particular the temporal horn of the lateral ventricle and the choroid fissure, on both sides. The MTA score ranges from 0 (no atrophy) to 4 (severe atrophy). MTA scores of the right and left hippocampi were added up in each patient. Furthermore, the scores were dichotomised into a normal value, with a grade 0 or 1 in each hippocampus, or an abnormal value with at least grade 2 in one hippocampus. The MTA score was estimated as part of routine patient care by two trained raters, who were blinded to the clinical information.

### *Neuropsychological measurements*

From the neuropsychological evaluation we used the data of the visual association test (VAT)<sup>13</sup> as a measure of episodic memory. The VAT was administered in 33 out of 39 patients. The material of the VAT consists of six association cards showing two interacting objects and six cue cards showing only one of the objects. Recall is tested without delay. The maximum score of the VAT is 6 points. An impaired score on the VAT was defined as a score  $< 5$ . The VAT was

dichotomised into a normal score ( $VAT \geq 5$ ) and an abnormal score ( $VAT < 5$ ). The verbal fluency (the ability to name as many animals as possible within 1 minute) was measured in 37 patients and used as a measure of language function. Furthermore, Trailmaking test A (TMT A, 32 patients) and Trailmaking test B (TMT B, 29 patients) were used as measures of executive function. Fluency, TMT A, and TMT B were corrected for age, sex, and education and expressed as z-scores on the basis of a reference population of cognitively normal subjects (Visser et al., 2000). Impairment on the fluency, TMT A, and TMT B was defined as a z-score below -1.28 (corresponding with a score below the 10<sup>th</sup> percentile). The sign of the z-scores of the TMT A and B was inverted such that a negative z-score indicated below average performance. Median time between LP and neuropsychological evaluation was 1.5 months (range 0-10).

#### *PAS scoring*

The PAS consists of six markers of AD: age, MMSE score, functional impairment, cognitive test performance, MTA score and Apo E genotype. Increasing PAS scores indicate a higher risk for AD. The PAS item 'functional impairment' was scored with the Global Deterioration Scale (GDS) (Reisberg et al., 1982). The PAS item 'cognitive test performance' was obtained with the VAT, fluency, and TMT B measures using the cut-off scores described above. Medial temporal lobe (MTL) atrophy was rated using the MTA scale as described above. Complete PAS scores were available for 26 subjects. The PAS and scoring instructions can also be found at [www-np.unimaas.nl/scales/pas/](http://www-np.unimaas.nl/scales/pas/).<sup>29</sup>

#### *Statistical analysis*

For statistical analysis, SPSS version 11.0 was used. On the basis of abovementioned cut off values for CSF A $\beta$ 42 and tau, MCI patients were divided into three groups at different risk for AD: a high-risk biomarker profile (low CSF A $\beta$ 42 and high CSF tau), an intermediate risk biomarker profile (low CSF A $\beta$ 42 and normal CSF tau *or* normal CSF A $\beta$ 42 and high CSF tau), and a low-risk biomarker profile (normal CSF A $\beta$ 42 and CSF tau). Mann Whitney U test (continuous variables) or Chi-square test with continuity correction (dichotomous variables) was used to test group differences. Correlations between the biomarkers CSF A $\beta$ 42 and tau versus gender, age, disease duration, MMSE, VAT, TMT A, TMT B, Fluency, MTA and PAS were calculated using the Spearman correlation coefficient. Statistical significance was set at  $p < 0.05$ .

## Results

Twenty-one (54%) of the 39 MCI subjects had a high-risk biomarker profile, 10 subjects (26%) had an intermediate risk biomarker profile (low A $\beta$ 42 (n=4) or high tau (n=6)), and 8 (20%) had a low-risk biomarker profile (Table 1, Figure 1). Median values of A $\beta$ 42 and tau in the high-risk biomarker group were comparable with the values from AD patients (data not shown). Compared to subjects with a low-risk biomarker profile, patients with a high-risk biomarker profile had more impaired TMT A and higher PAS scores (Table 1). Furthermore, they tended to have more impaired VAT and higher MTA scores. The intermediate biomarker profile group had higher PAS scores and lower MMSE and more impaired VAT scores, and a tendency towards higher tau levels and more impaired TMT A scores compared to the low-risk biomarker profile group (Table 1). No differences in neuropsychological and imaging markers existed between the high-risk and the intermediate risk biomarker profile group (Table 1). Furthermore, there were no differences in age, sex, disease duration, MMSE, and Apo E genotype between the three groups. In the whole MCI sample, A $\beta$ 42 levels correlated with tau levels, with MTA score, and with the PAS score. Tau levels correlated with the score on the VAT (Table 2). There were no correlations between the levels of A $\beta$ 42 and tau with disease duration or MMSE. Furthermore, there was no correlation between VAT and MTA score ( $R = -0.19$ ,  $P=0.33$ ).

## Discussion

About half of the MCI patients included in this cross-sectional study had a high risk of developing AD according to their biomarker profile at baseline. Twenty-six percent had either a low CSF A $\beta$ 42 and normal CSF tau level or a normal CSF A $\beta$ 42 and a high CSF tau level, while 20% had both normal CSF A $\beta$ 42 and tau levels. Subjects with a high-risk biomarker profile did not differ from subjects with an intermediate risk biomarker profile with respect to other markers of AD, while both high-risk and intermediate risk subjects differed significantly from the low-risk group in neuropsychological markers and PAS scores.

**Table 1 Patient characteristics and clinical variables**

|   | High risk<br>(N=21)<br>(Group 1) | Intermediate risk<br>(N=10)<br>(Group 2) | Low Risk<br>(N=8)<br>(Group 3) | P-value<br>Group 1 vs<br>Group 2 | P-value<br>Group 1 vs<br>Group 3 | P-value<br>Group 2 vs<br>Group 3 |
|---|----------------------------------|--|--------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Age   | 70 (53-81)                       | 69 (58-80)                               | 75 (56-78)                     | 0.61                             | 0.49                             | 0.42                             |
| Sex (F/M)                                       | 11/10                            | 6/4                                      | 2/6                            | 0.99                             | 0.31                             | 0.31                             |
| Duration of<br>cognitive<br>complaints<br>(yrs) | 3 (0-11)                         | 3 (1-10)                                 | 2 (1-6)                        | 0.64                             | 0.37                             | 0.22                             |
| MMSE  | 26 (23-30)                       | 26 (24-28)                               | 28 (23-29)                     | 0.17                             | 0.32                             | <b>0.04</b>                      |
| A $\beta$ 42<br>(pg/mL)                         | 395 (142-479)                    | 527 (241-1223)                           | 767 (504-916)                  | <b>0.03</b>                      | <b>&lt;0.001</b>                 | 0.37                             |
| Tau<br>(pg/mL)                                  | 707 (379-1108)                   | 410 (260-1545)                           | 262 (145-355)                  | <b>0.006</b>                     | <b>&lt;0.001</b>                 | 0.07                             |
| $\geq 1$ $\epsilon$ 4 allele<br>(%)             | 78                               | 40                                       | 43                             | 0.11                             | 0.23                             | 0.99                             |
| MTA R+L<br>MTA score<br>abnormal (%)            | 3.5 (2-5)<br>61                  | 3 (0-6)<br>56                            | 2 (0-4)<br>29                  | 0.48<br>0.99                     | 0.09<br>0.31                     | 0.42<br>0.57                     |
| VAT<br>< 5 (%)                                  | 2.5 (0-6)<br>82                  | 1 (0-4)<br>100*                          | 5 (1-6)<br>38                  | 0.17                             | 0.06<br>0.08                     | <b>0.01</b><br><b>0.04</b>       |
| Fluency<br>z < -1.28 (%)                        | 15 (11-33)<br>42                 | 18 (11-23)<br>22                         | 17 (10-24)<br>25               | 0.68<br>0.55                     | 0.87<br>0.61                     | 0.69<br>0.99                     |
| TMT A<br>z < -1.28 (%)                          | 52 (34-97)<br>39                 | 64 (34-82)<br>50                         | 37 (27-55)<br>14               | 0.86<br>0.99                     | <b>0.04</b><br>0.43              | 0.08<br>0.43                     |
| TMT B<br>z < -1.28 (%)                          | 156 (70-486)<br>88               | 150 (75-300)<br>67                       | 108 (65-249)<br>57             | 0.66<br>0.57                     | 0.14<br>0.28                     | 0.32<br>0.99                     |
| PAS   | 8.1 (3-11)                       | 8.2 (7-11)                               | 5.8 (4-7)                      | 0.74                             | <b>0.02</b>                      | <b>0.02</b>                      |

MTA R+L = medial temporal lobe atrophy right and left, VAT = visual association test, TMT A = trail making test A, TMT B = trail making test B, PAS = Preclinical Alzheimer's disease Scale. Groups 1, 2 and 3 are defined on the basis of the optimal cut off values for A $\beta$ 42 and tau comparing AD vs controls. Group 1: A $\beta$ 42  $\leq$  494 and tau > 356; Group 2: A $\beta$ 42  $\leq$  494 or tau > 356; Group 3: A $\beta$ 42 > 494 and tau  $\leq$  356. Disease duration is defined as the time in years between the first symptoms (by history) and the lumbar puncture. Values are expressed as medians (minimum-maximum) or percentages when dichotomised. MTA score was abnormal when at least one hippocampus had a score of  $\geq 2$ . \*: VAT was available in only 7 out of 10 patients, all with a value of < 5. Impairment on the fluency, TMT A, and TMT B was defined as a z-score below -1.28.

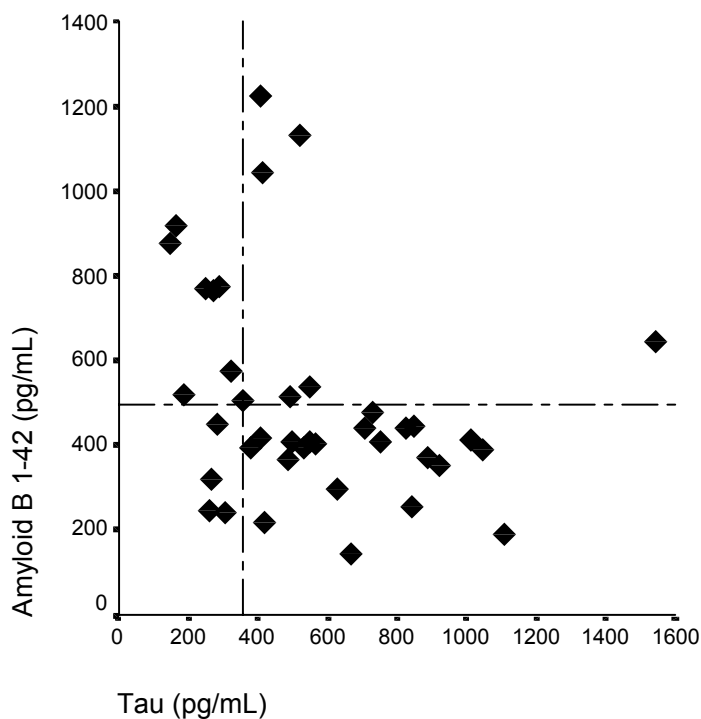
Furthermore, low CSF A $\beta$ 42 levels were associated with high MTA and high PAS scores, while high CSF tau levels were associated with low memory scores.

The high percentage of subjects with a high-risk biomarker profile is consistent with the high conversion percentage to AD in subjects with amnesic MCI.<sup>12,17</sup> The increased risk for AD in this group is further corroborated by the high frequency of other markers of AD such as the ApoE- $\epsilon$ 4 allele, memory impairment, and MTL atrophy. The findings in the group of patients with an intermediate risk biomarker profile are intriguing. Since there were no differences in clinical characteristics between the high-risk versus the intermediate risk group it seems likely that these subjects also have an increased risk for AD, even though one of the biomarkers was still in the normal range. Which biomarker is changed first in the disease process is not clear yet, as contradictory findings were reported by various studies describing either an increased CSF tau<sup>6,14,32</sup> or decreased CSF A $\beta$ 42<sup>23</sup> at baseline.

Another possible explanation for the intermediate risk could be that some of the patients will develop another type of dementia, for example frontotemporal lobar degeneration, in which a high CSF tau or a low CSF A $\beta$ 42 can be found.<sup>21</sup> Adding phosphorylated tau to the panel of biomarkers might help in differentiating MCI patients at risk for AD or other types of dementia.<sup>5</sup>

About 20% of the patients had a low-risk biomarker profile. These subjects also had less often other AD markers compared to patients with a high or intermediate-risk biomarker profile. This underlines the fact that these subjects indeed might have a low risk for the development of future AD. Furthermore, the percentage of 20% fits well with the results of a long-term study showing that about 20% of the MCI subjects do not progress to AD<sup>21</sup>, but clearly, longitudinal studies are necessary to determine whether these subjects will remain non-demented. The PAS score was the variable that was found to differentiate best between high/intermediate and low-risk biomarker profile groups. Since MCI is a heterogeneous disorder it is not surprising that the PAS, which combines a number of tests, differentiates better between MCI subjects at different risk for AD according to their biomarker profile than a single test.

**Figure 1 Plot A $\beta$ 42 and tau**



The dotted lines represent optimal cut off values obtained by comparing AD patients (N=92) with controls (N=38) (cut off value for A $\beta$ 42  $\leq$  494 pg/mL; cut off value for tau  $>$  356 pg/mL). Squares indicate MCI patients.

**Table 2 Correlations between biomarkers, VAT, MTA and PAS score**

|     | A $\beta$ 42 | Tau     |
|-----|--------------|---------|
| VAT | 0.26         | -0.44** |
| PAS | -0.43*       | 0.28    |
| MTA | -0.39*       | -0.03   |
| Tau | -0.34*       | --      |

MTA = atrophy score of medial temporal lobe; VAT = visual association test; PAS = Preclinical Alzheimer's disease Scale. \*P < 0.05, \*\*P < 0.01.

The increase of CSF tau is supposed to be the result of release from dying neurons containing a large number of neurofibrillary tangles (NFT), an important hallmark of AD. Our finding of a correlation between CSF tau and episodic memory impairment, as measured by the VAT, is consistent with clinicopathological studies<sup>8,10</sup>, in which a strong correlation between memory impairment with NFT densities and neuron numbers in the entorhinal cortex and hippocampus was found. As CSF tau is associated with neuronal loss one would also expect an association with the MTA score, but we did not find such a correlation. This finding is consistent with another study showing no association between CSF tau levels and global brain atrophy at baseline.<sup>31</sup> It is possible that the increase of CSF tau in our MCI population could be the result of neuronal loss in the entorhinal cortex or hippocampus at the very early stage of the disease, which may precede MTL atrophy as rated visually on MRI. A future study comparing CSF tau with the size of the hippocampus or entorhinal cortex as measured by volumetry could provide additional information about the cross-sectional relation between CSF tau and imaging markers of AD. It is also possible that tau is more a ‘state marker’, reflecting the intensity of the neuronal damage and degeneration.<sup>4</sup> Evidence for this explanation comes from a recent study showing an association between higher CSF tau levels at baseline and more rapid progression of atrophy after 1-year follow-up.<sup>31</sup>

A $\beta$ 42 is thought to be decreased in CSF as a result of deposition in senile plaques. In various studies, only a weak or no correlation between amyloid burden in the brain and cognitive status was found.<sup>3,8</sup> The absence of an association between decreased CSF A $\beta$ 42 and neuropsychological measurements in the present study is in line with this. The observation that lower CSF A $\beta$ 42 levels were associated with more atrophy in the medial temporal lobe is consistent with two other studies<sup>22,31</sup>, showing a correlation between decreased CSF A $\beta$ 42 and atrophy of the whole brain, ventricles or temporal lobe in patients with MCI and AD. CSF A $\beta$ 42 may thus be considered as a ‘stage marker’<sup>4,31</sup>, indicating the presence and severity of the disease at a certain stage and reflecting the total brain damage that has occurred since the onset of the disease. The correlation between CSF A $\beta$ 42 and the PAS score at baseline in MCI could be a reflection of both markers being independent predictors of AD, which was confirmed for A $\beta$ 42 in a recent study.<sup>11</sup>



A limitation of our study was the small cohort we included with the risk of selection bias and insufficient power to draw firm conclusions. Between January 2001 and November 2003 we collected CSF from approximately one third of all amnesic MCI patients seen at the Alzheimer Center, VUMC. The patients who underwent a LP were significantly younger compared to the patients without a LP, which can be explained by the inclusion criteria since we included only patients younger than 85 years. Cognition as measured by the MMSE was similar between the two groups. In addition, the degree of memory impairment and hippocampal atrophy, as well as the presence of  $\geq 1$   $\epsilon 4$  allele comparing the high-risk to the low-risk biomarker profile group correspond very well with those found in a recent population study when comparing MCI patients with controls and AD patients.<sup>9</sup> This makes us confident that no selection bias has occurred and that our cohort is representative of a 'normal' MCI group.

The relative high percentage of one or more  $\epsilon 4$  alleles in the three groups –even in the low-risk group- may be explained by the fact that all MCI patients could have an increased risk of AD, as was supported by the study of Smith et al.<sup>24</sup>, who also found a prevalence of 40% of the  $\epsilon 4$  allele in their MCI cohort. Both Apo E genotype and memory impairment are indicator markers for AD in MCI.

Our study underscores the recognition that MCI is a heterogeneous group of patients containing various biomarker profiles. MCI patients with a high- and intermediate-risk biomarker profile for AD also differed in other indicator markers from patients with a low-risk biomarker profile, adding to the biological validity of these markers. In addition, the relation between the biomarkers and other AD markers suggests that the markers are not independent at baseline. Longitudinal studies are necessary to interpret the correlations between biomarkers, imaging, and neuropsychological markers as well as their contribution to the diagnosis or as predictor of the severity of developing AD in the pre-dementia stage.

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## **CHAPTER 7**

### **CEREBROSPINAL FLUID AND MAGNETIC RESONANCE IMAGING MARKERS INDEPENDENTLY CONTRIBUTE TO THE DIAGNOSIS ALZHEIMER'S DISEASE**

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## **Abstract**

*Background:* Decreased amyloid  $\beta$  (1-42) ( $A\beta_{42}$ ) and increased (phosphorylated) tau in cerebrospinal fluid (CSF) are considered to be a reflection of plaques, tangles, and neuronal degeneration in Alzheimer's disease (AD). Atrophy of the medial temporal lobe (MTA) on magnetic resonance imaging (MRI) reflects neuronal loss in this area.

*Objective:* To compare diagnostic accuracy of CSF biomarkers and MTA in AD versus controls.

*Methods:*  $A\beta_{42}$ , tau and tau phosphorylated at threonine 181 (Ptau-181) were measured in CSF from 61 AD patients and 32 controls by sandwich enzyme-linked immunosorbent assay. A CSF biomarker profile for AD was constructed. MTA was rated visually on MRI.

*Results:* When AD patients and controls were evaluated separately, no correlations were present between the CSF markers and MTA score. Both MTA and CSF biomarker profile were independently associated with the diagnosis AD (MTA: OR (95% CI) = 28 (3 – 239); CSF biomarker profile: OR (95% CI) = 57 (13 – 262)). Among individuals younger than 65 years old and without MTA 60% suffered AD, and the finding of an abnormal CSF biomarker profile was limited to AD patients only.

*Conclusions:* MTA and CSF biomarkers seem to be of incremental value for the diagnosis AD. CSF analysis is most sensitive in the absence of MTA, and especially among early-onset AD patients.

## Introduction

Decreased cerebrospinal fluid (CSF) amyloid  $\beta$  (1-42) (A $\beta$ 42) in Alzheimer's disease (AD) is associated with intracerebral deposition of neuritic plaques, mainly composed of A $\beta$ 42 [2,31]. Increased CSF tau protein (tau) is considered to be a reflection of neuronal degeneration, caused by intraneuronal accumulation of neurofibrillary tangles containing (phosphorylated) tau.<sup>2,6,7</sup> Especially CSF phosphorylated tau (Ptau) is increased in AD compared to other types of dementia.<sup>2,27</sup> Although the combination of CSF A $\beta$ 42, tau and Ptau provides reasonable accuracy in the differentiation of AD from controls CSF concentrations of these biomarkers do overlap between groups.<sup>16,27</sup>

Atrophy of the medial temporal lobe (MTA) on magnetic resonance imaging (MRI) has been found to be an early and sensitive marker for AD<sup>37</sup> and is assumed to reflect underlying neuronal loss of the hippocampus and the temporal lobe. However, MTA may also be present in other types of dementia and absence of MTA does not exclude the diagnosis AD, especially in the early stages.<sup>37</sup> The latter particularly applies to early-onset AD (EAD), where MTA is often not prominently present.<sup>13,20</sup>

Relatively little is known about the association between MTA and CSF markers, while both are presumed to reflect Alzheimer pathology. From post mortem work it is known that hippocampal volume is a good indicator of the amount of plaques and tangles deposition<sup>8,14,30</sup>, which led us to suspect an association with CSF A $\beta$ 42 and (P)tau. An association between MTA and (P)tau would provide further evidence for the notion that both are measures of neuronal degradation or neuronal loss.<sup>3,9,18</sup> In addition, while MTA has become a widely used marker in AD diagnosis, CSF markers still have to be established as such. The aim of this study was twofold: first, to study the association between CSF markers and MTA, to provide insight whether both types of markers reflect the same neuropathological substrate. The second aim was to investigate whether both disease markers are independent predictors for the diagnosis AD. Therefore, we studied the relations between CSF A $\beta$ 42, tau, and Ptau with MTA in AD and controls. Furthermore, diagnostic accuracy of MTA and the combination of CSF A $\beta$ 42, tau and Ptau was compared in AD versus controls.



## Methods

### *Participants*

Sixty-one AD patients and 32 controls younger than 85 years old were recruited consecutively at the memory clinic of the Alzheimer Center VU University Medical Center, Amsterdam. All patients underwent a standardized diagnostic work-up, including medical history, informant interview, physical and neurological examination, screening laboratory tests, EEG, and Magnetic Resonance Imaging (MRI). The majority of patients underwent neuropsychological testing. Diagnoses were made in conference by a team of neurologists, neuropsychologists, a neurophysiologist, a psychiatrist and a radiologist. Diagnosis of probable AD was made according to the NINCDS-ADRDA criteria.<sup>23</sup> Although brain MRI contributed to the diagnostic process, it should be noted that MTA scores were not used. All clinicians participating in the diagnostic conference meeting were blinded to CSF results. Clinical diagnoses were revisited and confirmed after a minimum follow up period of 1 year and used in the analysis as gold standard. Disease duration was defined as the time in years since the first symptoms by history. The Mini-Mental State Examination (MMSE) was used as a measure of global cognitive function.<sup>11</sup> In addition, memory function was assessed using the visual association test.<sup>22</sup>

The control group of 32 subjects included 25 subjects with subjective memory complaints and three subjects with a positive family history for AD. These subjects presented at our memory clinic and underwent exactly the same diagnostic work-up as the AD patients. Patients were considered to have subjective memory complaints if all clinical investigations were normal. Additionally, four healthy spouses of patients without memory complaints were included. None of the controls had developed dementia after a follow up period of 1 year. The study was approved by the ethical review board of the VUMC. All subjects gave written informed consent to participate in the study.

### *Apo E genotype*

DNA was isolated from 10 ml EDTA blood and was available from 57 AD patients, and 31 controls. Apolipoprotein E (Apo E) genotype was determined with the light cyclers Apo E mutation detection method (Roche diagnostics GmbH, Mannheim, Germany). Patients were divided into two groups according to the absence or presence of  $\geq 1$  ApoE  $\epsilon 4$  alleles.

### *CSF analysis*

CSF was obtained by lumbar puncture (LP) between the L3/L4 or L4/L5 inter-vertebral space, and collected in 12 mL polypropylene tubes. After centrifugation at 3000 rpm for 10 minutes at 4°C, CSF was aliquoted in polypropylene tubes of 0.5 or 1 mL, and stored at -80°C until analysis. CSF A $\beta$ 42, tau and tau phosphorylated at threonine-181 (Ptau-181) were measured by commercially available sandwich ELISAs (INNOTEST™  $\beta$ -amyloid [1-42], INNOTEST™, hTAU-Ag and INNOTEST™ Phosphotau<sub>(181P)</sub>).<sup>33-35</sup> The optimal cut off values for CSF A $\beta$ 42 were set at data obtained in earlier studies, in which we applied a sensitivity of  $\geq 85\%$  for each individual biomarker in accordance with the Ronald and Nancy Reagan Consensus report after drawing Receiver Operating Characteristics curves.<sup>7,27,28</sup> The following cut off values were used: CSF A $\beta$ 42 < 495 pg/mL, CSF tau > 356 pg/mL and Ptau-181 > 54 pg/mL.<sup>27,28</sup> Based on the three markers, a CSF biomarker profile was constructed. The CSF profile was defined as abnormal when in addition to abnormal CSF A $\beta$ 42, also tau and/or Ptau were abnormal. The CSF biomarker profile was normal when CSF A $\beta$ 42, CSF tau and Ptau-181 were all in the normal range.

### *MRI analysis*

MRI scans were made on a 1.0 (N = 44 AD and 26 controls) or 1.5 (N = 17 AD and 6 controls) Tesla scanner (Siemens), and included a coronal T1-weighted 3D inversion-prepared gradient echo-sequence (168 slices, FOV 250mm, matrix 256x256; slice thickness 1.5 mm, in-plane resolution 1 mm). Mean (SD) time between LP and MRI scan was 2 (2.8) months in both AD and controls. The MTA score was rated visually according to the method described earlier.<sup>37</sup> The MTA score is based on a visual estimation of the volume of the medial temporal lobe, including the hippocampus proper, dentate gyrus, subiculum, and parahippocampal gyrus, and the volume of the surrounding CSF spaces, in particular the temporal horn of the lateral ventricle and the choroid fissure, on both sides. The MTA score ranges from 0 (no atrophy) to 4 (severe atrophy). MTA scores of the left and right hippocampus were averaged.<sup>21</sup> MTA scores were dichotomized, and an average MTA  $\geq 1.5$  was considered abnormal (requiring a score of 2 at least on one side). One rater (FB), who was blinded to the clinical information, performed the MTA ratings, with good intra-rater agreement for the MTA score.<sup>36</sup>

### *Statistical analysis*

SPSS version 11.0 was used. Mann Whitney U test was used to compare medians of clinical, CSF and MRI variables between AD and controls. Chi-square test was used to compare frequencies between groups. To determine associations between CSF biomarkers and MTA Spearman correlation coefficient was used. To estimate the impact of the different variables on diagnosis, logistic regression analysis was used, with diagnosis as dependent factor, CSF biomarker profile and MTA as independent factors, and age and gender as covariates. Apo E genotype was additionally corrected for in a separate model. Odds ratios (OR) with accompanying 95% confidence intervals (CI) are presented. Statistical significance was set at  $p < 0.05$ .

### **Results**

AD patients and controls were well matched for age and gender (Table 1). There were no differences between groups in the prevalence of history of hypertension, diabetes mellitus, or myocardial infarction ( $p > 0.10$ ). Median disease duration in AD was 4 years (range 0.5-11 years), median MMSE was 20 (9-28) in AD and 29 (25-30) in controls ( $p < 0.001$ ). CSF A $\beta$ 42 levels were decreased and CSF tau and Ptau-181 levels were increased in AD patients compared to controls. MTA score was significantly higher in AD patients compared to controls. The prevalence of Apo E  $\epsilon$ 4 was higher among patients with AD than among controls. In 37 AD patients, disease started before the age of 65 years (= early onset AD, EAD).

Table 2 shows characteristics of patients with early onset compared to patients with late onset AD. Patients with EAD had comparable disease severity as measured using the MMSE. In addition Apo E  $\epsilon$ 4 prevalence and level of CSF biomarkers were comparable among groups. Patients with late onset AD had more MTA than patients with EAD.

**Table 1 Subject characteristics and CSF and MRI analyses**

|                      | AD (N=61)     | Controls (N=32) | P-value |
|----------------------|---------------|-----------------|---------|
| Age (years)          | 66 (53-82)    | 64 (45-83)      | 0.43    |
| Gender (M/F)         | 28/33         | 16/16           | 0.71    |
| ≥ 1 Apo E ε4 (%)     | 44 (77%)      | 15 (48%)        | 0.006   |
| CSF Aβ42 pg/mL       | 324 (124-590) | 648 (232-1450)  | <0.001  |
| CSF tau (pg/mL)      | 632 (75-2615) | 256 (100-993)   | <0.001  |
| CSF Ptau-181 (pg/mL) | 78 (18-279)   | 48 (18-123)     | <0.001  |
| ≥ 1 Apo E ε4 (%)     | 44 (77%)      | 15 (48%)        | 0.006   |
| MTA score            | 1 (0-3.5)     | 0 (0-3)         | <0.001  |

Values are expressed as medians (range).

M = male, F = female; CSF = cerebrospinal fluid; Aβ42 = amyloid β (1-42); Ptau-181 = tau phosphorylated at threonine 181; MTA = medial temporal lobe atrophy.

In the whole group, there was a significant correlation between CSF Aβ42 and tau with MTA (CSF Aβ42 and MTA:  $r = -0.34$ ,  $p = 0.001$ ; CSF tau and MTA,  $r = 0.25$ ,  $p = 0.02$ ). CSF Ptau-181 did not correlate with MTA ( $r = 0.13$ ,  $p = 0.21$ ). When AD patients and controls were evaluated separately, no correlations were present between the CSF markers and MTA score (Figure 1). In AD, CSF Aβ42 was low in most patients, while there was variability in MTA score. CSF tau and Ptau-181 levels showed a large variation in AD patients. To assess whether MTA and CSF markers contributed independently to the diagnosis of AD, logistic regression analysis was performed. MTA as well as the CSF biomarker profile contributed to diagnosis

**Table 2 Characteristics of AD patients according to age-at-onset**

|                                      | Early onset (N=37) | Late onset (N=24) | P-value |
|--------------------------------------|--------------------|-------------------|---------|
| Age (years)                          | 59 (53-68)         | 74 (69-82)        |         |
| Gender (M/F)                         | 17/20              | 11/13             | 0.99    |
| MMSE                                 | 20 (9-28)          | 19.5 (10-28)      | 0.37    |
| Visual association test <sup>a</sup> | 3 (0 – 12)         | 3 (0 – 10)        | 0.77    |
| ≥ 1 Apo E ε4 (%)                     | 74%                | 83%               | 0.42    |
| CSF Aβ42 pg/mL                       | 307 (124-525)      | 342 (197-590)     | 0.36    |
| CSF tau (pg/mL)                      | 697 (75-2615)      | 533 (304-2605)    | 0.64    |
| CSF Ptau-181 (pg/mL)                 | 79 (18-279)        | 78 (46-254)       | 0.57    |
| MTA                                  | 1 (0-2.5)          | 1.5 (0-3.5)       | 0.01    |

Values are expressed as medians (range).

M = male, F = female; MMSE = mini-mental state examination, CSF = cerebrospinal fluid; Aβ42 = amyloid β (1-42); Ptau-181 = tau phosphorylated at threonine 181; MTA = medial temporal lobe atrophy. <sup>a</sup> available for 20 patients with early onset and 17 with late onset AD.

when entered separately in models with age and gender as covariates (MTA: OR (95% CI) = 14 (3 – 69); CSF biomarker profile: OR (95% CI) = 40 (11 – 143)). When both MTA and CSF biomarker profile were added in a model with age and gender, each disease marker contributed independently to AD diagnosis, with even higher odds ratios (MTA: OR (95% CI) = 28 (3 – 239); CSF biomarker profile: OR (95% CI) = 57 (13 – 262)). Additional correction for Apo E ε4 status only marginally affected the results (MTA: OR (95% CI) = 23 (3 – 193); CSF biomarker profile: OR (95% CI) = 46 (10 – 217)).

**Table 3 MTA and CSF biomarker profile in AD and controls according to age**

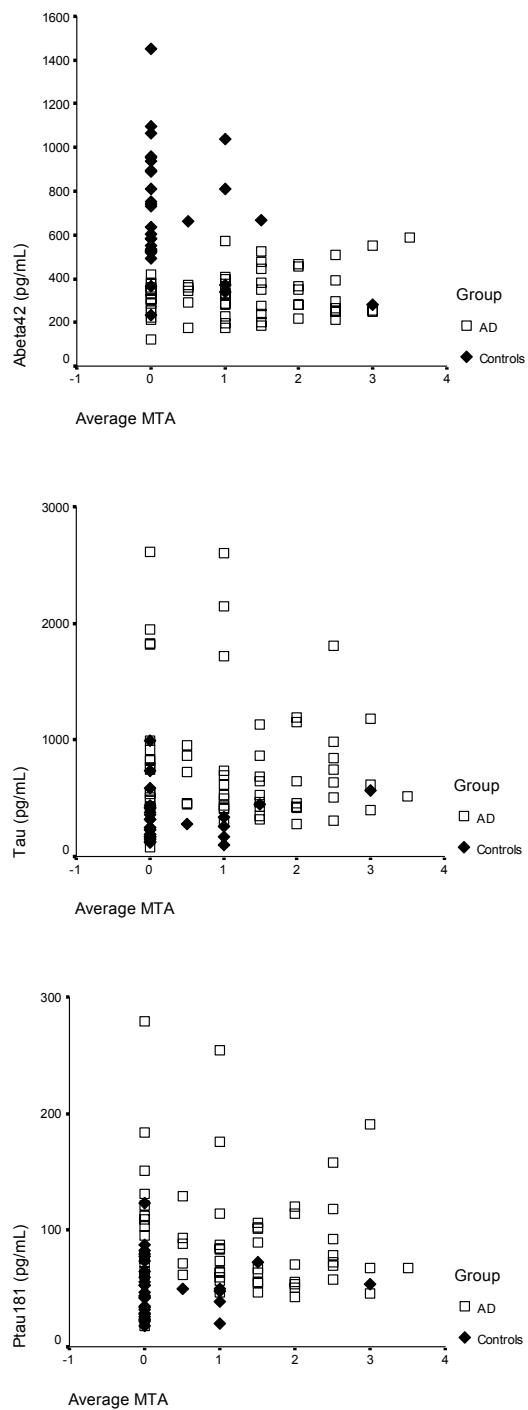
|             |                      | <65       |           | ≥65       |           |
|-------------|----------------------|-----------|-----------|-----------|-----------|
|             |                      | AD        | controls  | AD        | controls  |
| MTA absent  | Normal CSF profile   | 2 (5%)    | 16 (100%) | 1 (4%)    | 11 (69%)  |
|             | Abnormal CSF profile | 22 (60%)  | 0         | 8 (33%)   | 3 (19%)   |
| MTA present | Normal CSF profile   | 2 (5%)    | 0         | 4 (17%)   | 1 (6%)    |
|             | Abnormal CSF profile | 11 (30%)  | 0         | 11 (46%)  | 1 (6%)    |
| Total       |                      | 37 (100%) | 16 (100%) | 24 (100%) | 16 (100%) |

MTA = medial temporal lobe atrophy, CSF = cerebrospinal fluid. MTA absent = MTA score 0 or 1; MTA present = MTA score ≥ 2; normal CSF profile = Aβ42 > 494 pg/mL and tau < 357 pg/mL and Ptau-181 < 55 pg/mL; abnormal CSF profile = Aβ42 < 495 pg/mL and *either* tau > 356 pg/mL *or* Ptau-181 > 54 pg/mL.

When the analysis was restricted to controls and patients with mild AD only (MMSE ≥20, n=65), the contribution of MTA became less strong (OR (95% CI) = 10 (0.9 – 124)), while the CSF biomarker profile remained a significant predictor (OR (95% CI) = 37 (8 – 178)).

Visual inspection of younger and older patients separately (table 3), revealed that the majority of AD patients with early onset had no appreciable atrophy of the medial temporal lobe (65%; 24/37). Among individuals younger than 65 and without MTA, 60% (24/40) suffered AD (a priori). The finding of an abnormal CSF biomarker profile was limited to AD patients only, and therefore raised the a posteriori chance to 100%. Alternatively, among those younger than 65, all individuals with MTA were patients with AD, and therefore there was no added value of CSF analysis in the presence of MTA.

**Figure 1**



Scatterplots of medial temporal atrophy (MTA) by (a) amyloid  $\beta$  (1-42) (Abeta42), (b) tau and (c) tau phosphorylated tau at threonine 181 (ptau 181), respectively. Patients with AD are depicted by empty squares, and controls by filled diamonds. Within diagnostic groups, Spearman's correlations are not significant.

For individuals over 65 years of age, and without atrophy of the medial temporal lobe, the a priori chance to be an AD patient in our study was 39% (9/23). The additional finding of abnormal CSF biomarker profile raised this to an a posteriori chance of 73% (8/11). If MTA was present, an individual was likely to suffer AD (88%; 15/17). Additional finding of abnormal CSF raised this to an a posteriori chance of 92% (11/12).

## Discussion

In the present study, we could not demonstrate cross-sectional relations between CSF A $\beta$ 42, tau and Ptau-181 with MTA in AD or controls. Both MTA and the combination of CSF A $\beta$ 42, tau and Ptau-181 contributed independently to the diagnosis AD. Our data suggest that CSF analysis is most sensitive in the absence of MTA, and especially among younger patients.

Only a few studies investigated the cross-sectional relation between CSF biomarkers and atrophy on MRI in small groups of patients<sup>4,9,10,15,26,29,38</sup>, with conflicting results: some observed no relation between CSF tau and cerebral atrophy<sup>26,38</sup>, while others found a significant inverse relationship between CSF Ptau and hippocampal volume in subjects with mild cognitive impairment<sup>9,10</sup>, or, on the other hand, CSF tau levels corresponding to higher baseline hippocampal volume in AD.<sup>15</sup> Two studies showed lower CSF A $\beta$ 42 levels corresponding to lower brain volume<sup>38</sup> or volume of the temporal lobes.<sup>29</sup> The discrepancies between the different studies could be attributed to the selection of different patient groups as well as to the use of diverse methods to measure brain and hippocampal atrophy. The strength of our study is that we included a larger group of subjects than in former studies, in which we were able to compare all three biomarkers with MTA in subjects with AD and controls.

Our observation of higher CSF tau and Ptau concentrations in AD patients with relatively little MTA is in line with higher CSF tau and Ptau levels in patients with larger baseline hippocampal volumes mentioned in an earlier study.<sup>15</sup> Neuropathological studies have shown that neurofibrillary tangles precede entorhinal cortex (EC) and hippocampal neuronal loss.<sup>24</sup> The increase of CSF Ptau and tau might reflect the intensity of neuronal loss induced by the neuropathological changes<sup>38</sup>, which precedes volume loss as visualized on MRI.



Our finding of a relation between CSF A $\beta$ 42 and MTA in the whole group corresponds to the results presented elsewhere<sup>28,38</sup>, where the relation of CSF A $\beta$ 42 with cerebral atrophy in patients with a wide variation of cognitive impairment is attributed to CSF A $\beta$ 42 as stage marker of disease.<sup>28,38</sup>

Both MTA and CSF biomarkers independently contributed to the diagnosis of AD. With the abovementioned absence of direct cross-sectional correlations between the two disease markers one could consider that imaging- and CSF biomarkers reflect different stages of disease and/or another neuropathological substrate at one point in time. This might in part be due to the stable character of CSF biomarkers; CSF A $\beta$ 42, tau and Ptau levels demonstrate little change over time.<sup>1,9,19,32</sup> By contrast, MTA progresses with disease advancement, and the rate of atrophy is considered a valuable marker for disease progression.<sup>25</sup> Our data suggest that especially in the early stage of the disease, when cognitive scores are still high and when MTA is not yet prominently present, the value of CSF analysis is evident. CSF analysis may prove especially valuable in patients with EAD, which relatively often do not show MTA on MRI.<sup>12,13,20</sup>

One of the limitations of the present study is the cross-sectional design, in which the value of two different diagnostic methods was compared in subjects with the clinical diagnosis as gold standard. Furthermore, we cannot exclude an overestimation of the value of CSF biomarkers over the MTA score; the majority of the AD patients had an abnormal biomarker profile, while only half of the patients had considerable MTA. This might be due to the relatively higher proportion of subjects aged below 65 years old in this study, in which a LP is almost standard routine in our memory clinic. Alternatively, it is conceivable that MTA is not the most powerful measure in this group of patients. Other MRI-measures, such as whole brain volume or ventricle size should be evaluated in further studies.

Among the strengths of the study is the fact that only subjects with a follow-up period of at least one year were included, while a standardized diagnostic work up was used for every subject. In addition, MTA scores were assessed blinded for diagnosis. Although on visual inspection, our data suggest a difference in MTA score but not in CSF markers between AD patients with early and late onset disease -the latter is not congruent with a recent study<sup>17</sup> - further study with larger sample sizes is needed to properly assess these differences.

In conclusion, both CSF biomarkers and MTA seem to be of incremental value for the diagnosis AD. By applying both disease markers together, diagnostic accuracy could be increased. CSF biomarkers seem to be most promising in patients without appreciable MTA, which is common in early onset AD patients.

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## **CHAPTER 8**

### **GENERAL DISCUSSION**





The area of research on biochemical markers in AD is very broad and refers to the fundamental principles of the pathophysiology of AD as well as to the clinical utility. With this thesis we tried to provide some tools for how to use the three CSF markers A $\beta$ 42, tau and Ptau within the clinical context of a memory clinic of a secondary referral center. Before I come to the conclusion and future perspectives of this thesis, I will comment on the studies described in chapters 3-7.

## 8.1 Comments on chapters 3-7

### ***Chapter 3.2 Effects of processing and storage conditions on CSF amyloid $\beta$ (1-42) and tau concentrations in cerebrospinal fluid: implications for use in clinical practice***

According to the study described in **chapter 3.2** CSF A $\beta$ 42 and tau are stable in samples stored for several years at -80°C. The stability has been determined, however, by a protocol based on the Arrhenius equation. The validity of this method for the peptides CSF A $\beta$ 42 and tau should be confirmed in a real-time stability experiment. Furthermore, Ptau needs to be added to this panel of biomarkers. A very recent study confirmed our data<sup>29</sup>, and showed that CSF Ptau was the most stable marker of all three CSF markers. Since the end of 2000 we have collected CSF samples, but for follow up studies longer time intervals are needed with large collections of CSF pools.

Another important issue is that the variability of CSF A $\beta$ 42 among centers might well be attributable to the procedure of sample treatment in the first hours after collection. This issue has not been investigated in our study and needs to be considered and studied in multi-center studies. An international validity study has been started recently.<sup>30</sup>

The last point we would like to discuss here is the influence of internal factors -recovery rate and linearity- on the quantitative test results of the INNOTEST<sup>TM</sup>  $\beta$ -amyloid (1-42) ELISA (see also the Appendix for results from our laboratory). These findings, which might not be solely attributed to this particular ELISA, give concern about the robustness of the A $\beta$ 42 assay. Possibly, the same mechanisms might be involved as during repeated freeze/thaw cycles or when samples are stored at temperatures > -80°C, i.e. conformational changes in the fibrillary  $\beta$ -sheeted A $\beta$  peptide or masking of the epitope by binding to another protein.<sup>23</sup> Therefore, we also wonder what is exactly measured in the INNOTEST<sup>TM</sup>  $\beta$ -amyloid (1-42) ELISA; full length

peptide A $\beta$ 42 only or the full length peptide in combination with an A $\beta$  peptide, bound to a combination of other proteins, like -Apo J, Apo E,  $\alpha$ 1-antichymotrypsin and/or albumin?<sup>31,32</sup> In the studies by Wiltfang et al.<sup>23,32</sup> freezing prior to analysis influences the concentration of CSF A $\beta$ 42 as measured by SDS-PAGE/immunoblot, while a higher CSF A $\beta$ 42 concentration is measured by this technique compared to ELISA. This might be attributed to carrier mediated epitope masking, as SDS-heat denaturation may strip the A $\beta$ 42 peptide from the binding epitope as detected in the A $\beta$ 42 ELISA. Knowledge about whether we detect free or bound A $\beta$  peptide is very important applying CSF A $\beta$ 42 as marker of *progression* in longitudinal studies, especially when measuring the effect of anti-amyloid drugs. Furthermore, it needs to be taken into account in the differentiation of AD from other types of dementia, i.e. FTD and CJD, in which also decreased CSF A $\beta$ 42 concentrations are found.<sup>36</sup>

Nonetheless, the A $\beta$ 42 peptide concentration has been shown to be decreased in CSF from the majority of AD patients when measured by different laboratory techniques<sup>21</sup>, which makes the use of CSF A $\beta$ 42 as *diagnostic* marker for AD very promising.

**Chapters 4.1 and 4.2 Differences and similarities between two frequently used assays for Amyloid  $\beta$  42 in cerebrospinal fluid and Amyloid  $\beta$  38, 40, and 42 species in cerebrospinal fluid: more of the same?**

The main findings of the two studies are that absolute concentrations of CSF A $\beta$ 42 as measured by two distinct ELISAs are comparable and that CSF A $\beta$ 42 is selectively decreased in CSF as compared to the other two most prominent CSF A $\beta$  peptides, A $\beta$ 38 and 40. These results are encouraging for the implementation of CSF A $\beta$ 42 as diagnostic marker for AD in clinical practice, and seem to be irrespective of the ELISA used. Taking a closer look at the data, several issues need attention. The first issue is the binding characteristic of the antigen which may vary with temperature, buffer solutions and even among lots. Second issue is the variability of the affinity of the antibodies for the various CSF A $\beta$  peptides involved in the pathogenesis of AD. With this study we did not exchange calibrators and antibodies used in the two assays, i.e. 6E10 and 3D6 (see also **chapters 3.1 and 4.1**) as well as the different C-terminally directed antibodies. Innogenetics did study this issue to some extent<sup>33</sup>, and roughly the same results were found by a multiparameter xMap<sup>TM</sup> technology-based assay: CSF A $\beta$ 42 is decreased in AD as measured by

different N-terminally directed antibodies (6E10, 3D6 and 4G8), although diagnostic accuracy varied to some extent. The SELDI-TOF data reported by Innogenetics<sup>33</sup> and SDS-PAGE/Immunoblot data by Wiltfang<sup>23</sup> give a more refined impression of the different types of A $\beta$ 42 present in CSF, which may not only be promising for use in the early (MCI) stage of disease, but also for differentiation between different types of dementia, especially between AD and FTD. The tendency towards a difference in concentration between CSF A $\beta$  1-42 and A $\beta$  N-42 in FTD (*chapter 4.1*) is remarkable, and needs further investigation. By several research groups a putative role for beta amyloid metabolism disturbances in the pathogenesis of some types of FTD is found<sup>34,35</sup>.

The decrease of A $\beta$ 42 in CSF compared to the other C-terminally truncated A $\beta$  peptides, A $\beta$ 38 and 40, might be due to deposition of insoluble A $\beta$ 42 in the plaques has been stated in *chapter 4.2*. However, this is by far not clear yet. Another explanation might be that CSF A $\beta$ 42 is to a higher extent bound to another protein than CSF A $\beta$ 38 and 40 as has been shown before<sup>32</sup>. Evidence for this hypothesis is the decrease of CSF A $\beta$ 42 in Creutzfeldt-Jakob patients<sup>36</sup> in which no classic  $\beta$ -amyloid (neuritic) plaques are found in the brain<sup>37</sup> Here, a comparable (the same?) pathological chaperone might be involved as well.

#### ***Chapter 5.1 Amyloid $\beta$ 42 (1-42) and phosphorylated tau in CSF as markers for early onset Alzheimer's disease***

The combination of low CSF A $\beta$ 42 and high Ptau-181 yields the highest diagnostic accuracy in a cohort of EAD patients compared to FTD patients. This especially accounts for the specificity and for the negative likelihood ratio with two markers negative compared to one positive. These findings are most essential for clinical practice; a diagnostic marker is useful when it has a low false positive rate and when it can with great certainty rule out the presence of a disease. The sensitivity of the combination of CSF A $\beta$ 42 and tau did not achieve  $\geq 85\%$  in AD, which is needed for an ideal biomarker in accordance to the Reagan Consensus Report (see also Appendix).<sup>38</sup> In some EAD patients either CSF A $\beta$ 42 was low or Ptau-181 was high. This is probably due to the low cut off value applied for CSF A $\beta$ 42 comparing EAD with FTD. Moreover CSF A $\beta$ 42 is also decreased in a substantial proportion of the FTD patients (see also

*chapter 5.2*). Therefore, according to our results, the best single marker to differentiate EAD from FTD is CSF Ptau. On the contrary, CSF Ptau alone is found to be less specific than CSF tau and A $\beta$ 42 in controls with subjective memory complaints. Comparing AD with controls, CSF A $\beta$ 42 is the marker with the highest sensitivity for AD (*Chapter 5.1*).<sup>21,39</sup>

Until now three assays are available measuring CSF tau phosphorylated at different epitopes; phosphorylation at threonine 231 (Ptau-231), threonine 181 (Ptau-181), and serine 199 (Ptau-199). These markers demonstrate comparable diagnostic accuracy comparing AD with controls.<sup>40</sup> For the differentiation of AD from other types of dementia CSF Ptau-231 and Ptau-181 performed equally well (see also Appendix for our unpublished results comparing AD with FTD). Combinations of the three markers did not add to the discriminative power when compared to a single marker.<sup>40?</sup> Neuropathological data have shown that phosphorylation of tau at the three epitopes occurs at different stages of disease. Unfortunately, no difference in the prediction of the rate of cognitive decline between the different phosphorylated Ptau epitopes could be shown in MCI patients with 1 year clinical follow up.<sup>41</sup> A possible explanation for this finding might be the relatively short time of follow up, but also test characteristics might be involved: not the *absolute* quantification of the phosphorylation of tau at the specific epitope is measured in CSF, but the *presence* of Ptau phosphorylated at this particular epitope in CSF. In addition, little is known about CSF flow, kinetics and clearance dynamics and their influence on the concentration of (P)tau proteins.<sup>42</sup>

## ***Chapter 6 Biomarker profiles and their relation to clinical variables in mild cognitive impairment***

The findings of this study point to a relation of CSF A $\beta$ 42 and tau to clinical markers of AD in the MCI stage of disease. These results are very important, not only because they add to the biological validity of the markers, but they also support the added value of the biomarkers in selecting MCI patients for clinical studies. We divided the MCI patients into three groups according to their biomarker profile. The biomarker profile was assumed positive for AD when the CSF A $\beta$ 42 concentration was decreased and CSF tau was increased. Preliminary results show that 56% of our MCI cohort developed AD in a follow up period of 19 months.<sup>43</sup> Both CSF A $\beta$ 42 and tau were significantly associated with AD at follow-up comparing progressive with stable

MCI patients. These associations were also found when the markers were analyzed separately. These results justify our choice to divide the patients into three groups; high-, intermediate- and low-risk group. Overall, patients within the high- and intermediate- risk group were comparable for all clinical variables. Including CSF Ptau to the panel of biomarkers did not influence the results and conclusions (unpublished results). All patients with a high CSF tau also had a high CSF Ptau, except for one patient, who would therefore have an intermediate risk for AD instead of a low risk.

The different relations of CSF A $\beta$ 42 and tau with MTA, PAS and memory impairment is remarkable. However, our results are in line with those of another independent cohort study.<sup>44</sup> The cross-sectional associations between CSF tau and neuropsychological markers may very well be restricted to the MCI stage of disease. The VAT is an instrument that is changed very early in the disease process<sup>45</sup>, even before the clinical diagnosis of AD is obtained. Until now, it was not possible to find consistent relations between CSF biomarkers and neuropsychological markers in AD<sup>46</sup>, except for a relation between CSF Ptau-231 at baseline and annual rate of change in MMSE score in MCI.<sup>47</sup>

### ***Chapter 7 Cerebrospinal fluid and magnetic resonance imaging markers independently contribute to the diagnosis Alzheimer's disease***

Major finding of this study was that both CSF and MRI markers contribute to the clinical diagnosis AD. Both diagnostic markers are supposed to reflect neuropathology of AD.<sup>48-50</sup> All studies investigating hippocampal volumes with postmortem neuropathology have found reasonable associations with neuron loss as well as plaques and tangles densities.<sup>42,48,49</sup> Associational studies between biochemical markers and postmortem neuropathological data are very sparse and not conclusive.<sup>50,51</sup> Unfortunately, postmortem CSF tau-, Ptau- and A $\beta$ 42- concentrations seem not to be valuable to use for associational studies with neuropathological findings, as the CSF concentrations are totally different from antemortem CSF concentrations, with no distinction between AD and controls (unpublished results).<sup>52</sup> Several explanations for the absence of a relation between CSF biomarkers and MTA are described in the paper. Here, I would like to comment on the presumed differences in early onset AD versus late onset AD

(EAD vs LAD), as well as the inability to detect longitudinal changes in CSF A $\beta$ 42, tau and Ptau concentrations.

Although clinical presentation differs between EAD and LAD<sup>53</sup>, neuropathological findings are comparable. However, the distribution of neuronal loss is in proportion to the clinical picture from patients, especially in the early stage of disease; patients who present with focal signs like aphasia, apraxia, executive dysfunction or visual disturbances have predominantly focal degeneration of the brain, and in lesser extent hippocampal atrophy.<sup>54</sup> In our study we did not divide the AD patients into a memory versus a non-memory type according to their neuropsychological profile. This would have revealed whether differences between the EAD and LAD group with respect to discordance between CSF markers and MTA was due to fewer patients in the EAD group presenting with memory disturbances and thus with less MTA. Interesting in this light is the finding of a relationship between the clinical phenotype of EAD – either the amnesic phenotype or the non-memory phenotype- and the presence of  $\geq 1$  Apo E  $\epsilon$ 4 allele.<sup>55</sup> Furthermore, hippocampal atrophy is also considered to increase with age.<sup>56</sup> Patients with AD still tended to have higher MTA scores compared to age-matched controls (unpublished results, see also Appendix).<sup>57</sup> However, we cannot exclude the possibility that younger AD patients have less MTA than older AD patients due to the age factor.

Until now no longitudinal changes of CSF A $\beta$ 42, tau and Ptau could be demonstrated, except for CSF A $\beta$ 42 in one study with prolonged follow up.<sup>58</sup> If the CSF markers are supposed to reflect neuropathological changes in AD, one would expect a decrease of CSF A $\beta$ 42 and an increase of CSF tau and/or Ptau with time, as a result of increased plaque deposition and neuron loss, comparable to the increase of MTA with progression of disease. Probably more complex mechanisms are involved; one hypothesis might be that there is an increased total production of A $\beta$ 42 during progression of disease, not per se leading to a decreased but even to a steady or a paradoxically increased CSF A $\beta$ 42 concentration, which can only be detected after prolonged follow up. In addition, with disease progression less neurons are left to release (P)tau in CSF, which might result in steady or decreased CSF (P)concentrations. But, also CSF flow and clearance dynamics might be involved<sup>42,59</sup>, as well as ELISA characteristics like epitope masking of A $\beta$ 42, or the presence of various A $\beta$  peptides in CSF, which might have a different role in progression of disease. Taking all these considerations into account, it is not surprising that the

CSF biomarkers A $\beta$ 42, tau and Ptau, which are already changed before the clinical picture of AD becomes overt, do not have a relation with MTA, which increases as disease progresses.



## 8.2 CONCLUSIONS

The studies described in this thesis intended to assess whether the CSF biomarkers A $\beta$ 42, tau and Ptau can be used for early and differential diagnosis of AD. Findings from former studies were confirmed in a clinical setting, with the addition of some new insights: the combination of CSF A $\beta$ 42 and Ptau achieved the highest diagnostic accuracy for the differentiation of EAD from FTD; CSF biomarkers are not independent from other clinical parameters in the MCI stage of disease; and both CSF A $\beta$ 42, tau and Ptau and MTA are of incremental value for the diagnosis AD. Furthermore, the variability of CSF A $\beta$ 42 concentrations is explained by pre-analytical and internal (ELISA) factors. However, the commercial ELISAs are quite reliable as diagnostic method compared to the other methods available.

None of the biochemical markers is sufficiently accurate to be used as the sole diagnostic tool. Will there ever be one, as the gold standard for AD is still the clinical diagnosis? Without a doubt, more clinicopathological studies are needed, although it is not very likely that all clinically diagnosed AD patients will have a pure form of AD neuropathologically, which might be again confusing to interpret the CSF data. Be that as it may, the results of the present thesis led us to the conclusion that the three CSF biomarkers A $\beta$ 42, tau and Ptau can be used as diagnostic tool for AD in a secondary referral setting *within* the clinical context and only *in addition to* other diagnostic methods. Most promising are the CSF markers in the diagnostic evaluation of EAD. Furthermore, CSF A $\beta$ 42, tau and Ptau are potential markers for prediction of AD in MCI patients. Until now, CSF A $\beta$ 42, tau and Ptau do not appear to be good markers to track progression of disease. The absence of neuropathological confirmation of the diagnosis in most cases requires prolonged follow up time of the patients especially in the early stage of disease. Answers to the questions in **chapter 2** are summarized below. Recommendations or guidelines for the use of the CSF biomarkers in clinical practice are listed at the end of this paragraph (see also **chapter 1.2**):

*Are CSF samples stable as far as A $\beta$ 42 and tau are concerned?*

- CSF A $\beta$ 42 and tau are stable when stored for years at -80°C. CSF A $\beta$ 42 concentrations are influenced by storage temperatures > -80°C and after repeated freeze/thaw cycles.

CSF tau is stable under these conditions, except when stored for more than 10 days at 37°C.

*Are CSF concentrations of A $\beta$ 42 comparable when measured by two different ELISAs?*

- In AD and controls CSF A $\beta$ 42 concentrations are comparable when measured by two different ELISAs for A $\beta$ 42. In FTD, CSF A $\beta$ 42 concentrations as measured by the two ELISAs slightly differ, which suggest a role of  $\beta$  amyloid metabolism disturbances in FTD. Both ELISAs have a comparable diagnostic accuracy in AD versus controls.

*Is there a relation between various C-terminally truncated A $\beta$  peptides in CSF?*

- All three C-terminally truncated A $\beta$  peptides in CSF are interrelated in both AD and controls. CSF A $\beta$ 42 is selectively decreased in AD.

*What is the diagnostic value of CSF A $\beta$ 42, tau and Ptau in early onset AD versus frontotemporal dementia?*

- Diagnostic accuracy of the combination of CSF A $\beta$ 42 and Ptau-181 in a cohort of EAD and FTD patients is good, especially the specificity and negative likelihood ratio. CSF Ptau is a better marker for the differentiation of EAD from FTD than CSF tau.

*Are CSF A $\beta$ 42 and tau independent predictors of AD?*

- At baseline in MCI, CSF A $\beta$ 42 and tau have relations with other indicator markers of AD, i.e. MTA and memory loss. Furthermore, patients with a higher risk of AD according to their biomarker profile also perform less well on neuropsychological tests as well as the preclinical AD scale. These findings suggest that the CSF markers are not independent in the MCI stage of disease.

*What is the relation between CSF markers and medial temporal lobe atrophy?*

- No relation could be found between CSF markers and MTA in AD or controls. Both disease markers seem to reflect another neuropathological substrate at one point in time. Both disease markers contribute independently to the diagnose AD.

### **Guidelines for the use of CSF A $\beta$ 42, tau and Ptau in clinical practice**

1. Determine A $\beta$ 42, tau and Ptau in CSF when there is doubt about the diagnosis AD, and when MRI markers and neuropsychological findings are not conclusive
2. Determine A $\beta$ 42, tau and Ptau in CSF in presenile dementias, as the differential diagnosis here is wider and more complicated, and the existing diagnostic tools less sensitive
3. Determine A $\beta$ 42, tau and Ptau in CSF in the early stage of disease, in patients for whom treatment is being considered.
4. All three biomarkers CSF A $\beta$ 42, tau and Ptau should be measured in order to differentiate AD from subjects with subjective memory complaints as well as patients with other types of dementia
5. The biomarker profile is positive for AD when in addition to CSF A $\beta$ 42, also tau *or* Ptau are abnormal; i.e. at least two markers should be positive
6. Try to achieve postmortem verification of the diagnosis or otherwise prolonged follow up time
7. In case of a negative biomarker profile at baseline, repeat the CSF measurements when the patient clinically progresses to AD or MCI, preferably at least one year after the first LP

### **Laboratory guidelines for the use of CSF A $\beta$ 42, tau and Ptau**

1. Preferably, samples should be stored at -80°C immediately after collection
2. Baseline- and follow up- CSF samples should be analyzed after comparable storage conditions and within the same ELISA to avoid confounders by repeated freeze/thaw cycles or test characteristics
3. For longitudinal studies we recommend storing large collections of CSF pools, which can be used as control samples to measure degradation of biomarkers over time
4. As the median concentrations of CSF A $\beta$ 42, tau and Ptau differ between laboratories, we recommend to define internal cut off values until international validity studies are completed (see Appendix for reference values of the VUMC)

### 8.3 FUTURE PERSPECTIVES

In *chapter 2* ‘topics for future research’ are listed. In the present chapter I will mainly focus on how and which biomarkers could be used for tracking the progression of the disease, especially in the early stage of disease and in relation to promising therapies. Next, a brief commentary on the few studies about plasma markers for the (early) diagnosis of AD will be given.

Obviously, more longitudinal prospective studies are needed. CSF A $\beta$ 42 is a good marker to predict AD, even in asymptomatic elderly patients.<sup>60</sup> Very interesting in this light are the results of Fagan et al.<sup>61</sup>, in which a relation was found between in vivo brain amyloid, as detected by PET imaging of the amyloid-binding agent PIB, and decreased CSF A $\beta$ 42 levels in 7 patients, including 3 non-demented subjects. These findings however are not yet replicated by others. Results depend also on which type of A $\beta$ 42 peptide is measured: the N-terminally truncated forms of A $\beta$ 42, which might be indicator markers of the early stage of disease<sup>18,33</sup> or full length A $\beta$  1-42, which is decreased throughout the whole spectrum of disease. Then, oligomers of A $\beta$  peptides seem to be very promising for early detection of disease and possibly therapeutic monitoring, also because they are directly associated with memory loss due to their toxic effects on synapses.<sup>82</sup>

No change of CSF A $\beta$ 42 is found with progression of disease, but also not in relation to therapy, as has been shown in studies with acetylcholinesterase inhibitors<sup>62,63</sup>, and even after A $\beta$  immunization CSF A $\beta$ 42 remained the same.<sup>64</sup> Although concentrations of CSF A $\beta$ 40 are found to be comparable between AD and controls, there might be a role for this peptide in follow-up studies, as the peptide has been shown to be increased in CSF of MCI patients at follow up.<sup>28</sup> CSF A $\beta$ 38 might be interesting to study in relation to therapy, as this peptide has been shown to increase after treatment with non-specific anti-inflammation drugs (NSAIDs) in cells and mice studies, while amyloidogenic CSF A $\beta$ 42 is decreased, probably due to an allosteric effect on  $\gamma$ -secretase.<sup>65,66</sup> No studies are published yet investigating the effect of NSAIDs on CSF A $\beta$ 42 or 38 in humans, and only a trend was found to decreased plasma A $\beta$ 42 levels in NSAID users.<sup>67</sup> Recently a promising study is published, in which the effect of  $\gamma$ -secretase inhibitors on

reductions of CSF A $\beta$ 40 levels in mice is described.<sup>68</sup> While CSF A $\beta$ 42 is thus not a good marker to measure effect of therapeutics, A $\beta$  (auto) antibodies might be<sup>69</sup>; subgroup analysis suggests that patients who developed antibodies reactive to amyloid plaques after A $\beta$  immunization perform better on neuropsychological tests compared to patients who did not develop these antibodies.<sup>70</sup> However, all these studies are very preliminary and must be interpreted cautiously. The same implies for the decrease of CSF tau in 11 patients, who received A $\beta$  immunization, which might be due to reduced rate of cellular degeneration in patients who developed high titers of anti-AN1792 antibodies<sup>64</sup>, but also a dilutional effect of CSF tau might be involved, as ventricular enlargement and reduced brain volume is found in the antibody responders.<sup>71</sup> CSF Ptau-231 has been shown to decrease in AD with disease progression in one study.<sup>72</sup> Further studies are needed to investigate the value of CSF tau and Ptau to track progression of AD/MCI.

The plasma A $\beta$ 42 and A $\beta$ 40 markers are not useful for diagnosis, as has been shown by 8 studies.<sup>69</sup> A $\beta$ 42 is increased in plasma of affected and even unaffected family members with mutations in PSEN 1 and 2 and APP, as well as in patients with Down syndrome with and without dementia.<sup>73</sup> Probably this increase of plasma A $\beta$ 42 is a result of an overall increase in A $\beta$ 42 production, in the brain but also in peripheral sources such as blood.<sup>74</sup> There are findings pointing to an increase of A $\beta$ 42 in plasma of some patients long before and during the early stages of AD, with a decline thereafter.<sup>74</sup> The plasma A $\beta$ 42 marker might therefore be used to track patients at risk for AD (which might be patients with  $\geq 1$  Apo E  $\epsilon$ 4 allele and a strong positive family history), but only when followed for longer periods as the differences in frequency of AD by plasma A $\beta$ 42 emerge only after 2-3 years and the specificity of AD diagnosis is higher in more advanced stages.<sup>74</sup> Moreover, ethical issues are involved; until there is no absolute cure for AD the knowledge for the patient of having a biomarker profile indicative for AD is not warranted, making the use of A $\beta$ 42 as predictive (plasma or CSF) marker in most cases only a topic for research.

Isoprostanes are markers of oxidative stress and involved in a number of neurodegenerative disorders, including AD. It can be measured in CSF, plasma and urine, and is increased in AD and MCI in CSF.<sup>75,76</sup> Conflicting results are found in plasma concentrations of isoprostanes in

AD versus controls.<sup>75,77</sup> One study showed an increase of isoprostanes in CSF at follow up in MCI.<sup>42</sup> As diagnostic marker isoprostanes seem not be very useful as they lack specificity, but there might be possible role as marker of progression to dementia.

Conflicting results are also found in inflammatory markers measured in serum: some authors found an increase of interleukine 6 (Il-6) in serum of AD patients, while others could not replicate this finding.<sup>69</sup> The same accounts for  $\alpha$ 1-antichymotrypsine ( $\alpha$ 1-ACT) in serum.<sup>78</sup> These differences might be contributed to differences in assay methodology and sensitivity, and small sample sizes with heterogeneous patient and control populations. We could not find differences in Il-6 and  $\alpha$ 1-ACT in serum between AD and controls in our laboratory (unpublished results). In a collaboration study with investigators in Milan we could not demonstrate differences between the chemokines Interferon- $\gamma$ -inducible Protein-10 (IP-10), Interleukin-8 (IL-8), and Monocyte Chemotactic Protein-1 (MCP-1) in serum from AD, MCI and controls.<sup>79,80</sup> CSF appears to be a better fluid to investigate interleukins and chemokines, however, as differences were found in a cross-sectional study for CSF IP-10, IL-8 and MCP-1 in mild-moderate AD and MCI versus controls<sup>80</sup>, although the last two markers seem not to be specific for AD.<sup>81</sup> Follow-up studies in MCI might reveal whether these markers might be used for prediction or even progression of disease. Furthermore, additional studies are needed to confirm our unpublished data of increased CSF  $\alpha$ 1-ACT concentrations in AD versus controls as compared to the serum concentration. As  $\alpha$ 1-ACT is found at a very early stage of AD in the neuritic plaques, studies are underway to investigate the relation of  $\alpha$ 1-ACT and A $\beta$ 42 in CSF and whether it is possible to detect complexes of these two peptides in CSF.

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\* Chapters 1.1, 2, and 8.1-8.3. References used in chapters 3-7 are mentioned at the end of each paper.





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## **APPENDIX**



## ***Chapter 1.1***

### NINCDS-ADRDA criteria<sup>1</sup>:

#### Probable AD

- Dementia
- Clear conscience
- Dysfunction in two or more cognitive domains
- Progressive deterioration of cognitive dysfunction
- Age between 40 and 90 years old
- No signs of systemic disorder or other brain disease

#### Possible AD

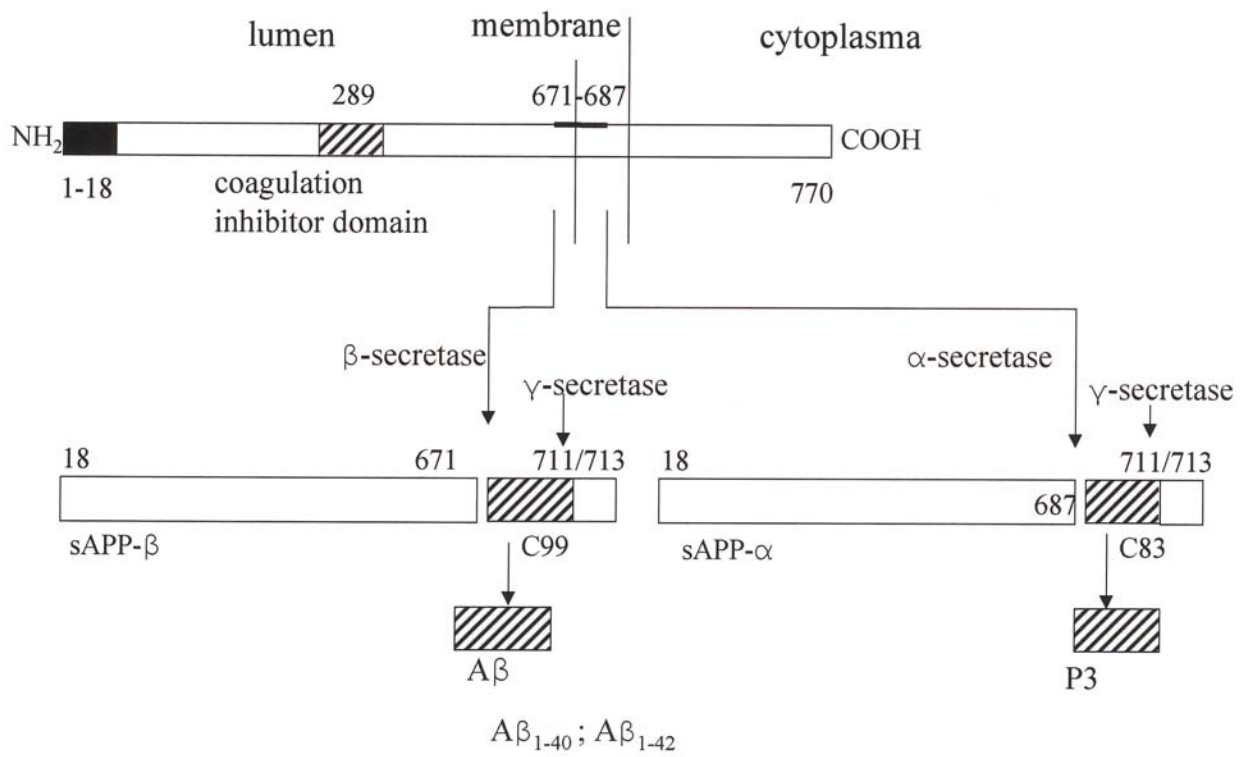
- Dementia of the Alzheimer type
- Presence of a systemic disorder or brain disease

### Amnesic MCI (Petersen et al. <sup>16</sup>) :

- Memory complaint
- Memory disturbances at neuropsychological screening tests
- No other cognitive dysfunction
- Intact function in daily activities
- No dementia

## Chapter 1.1

### Proteolysis of amyloid precursor protein (APP)<sup>87</sup>



### Chapter 3.3

Figure 1

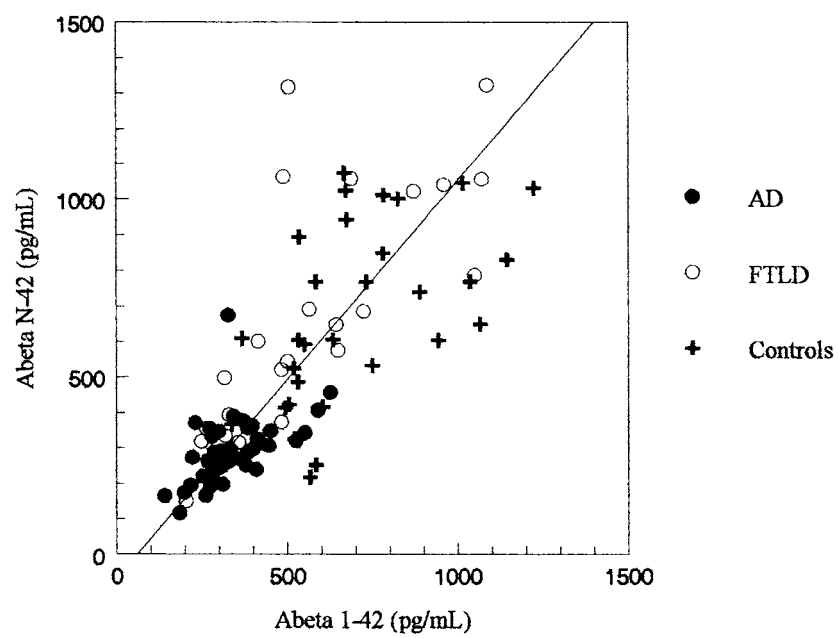
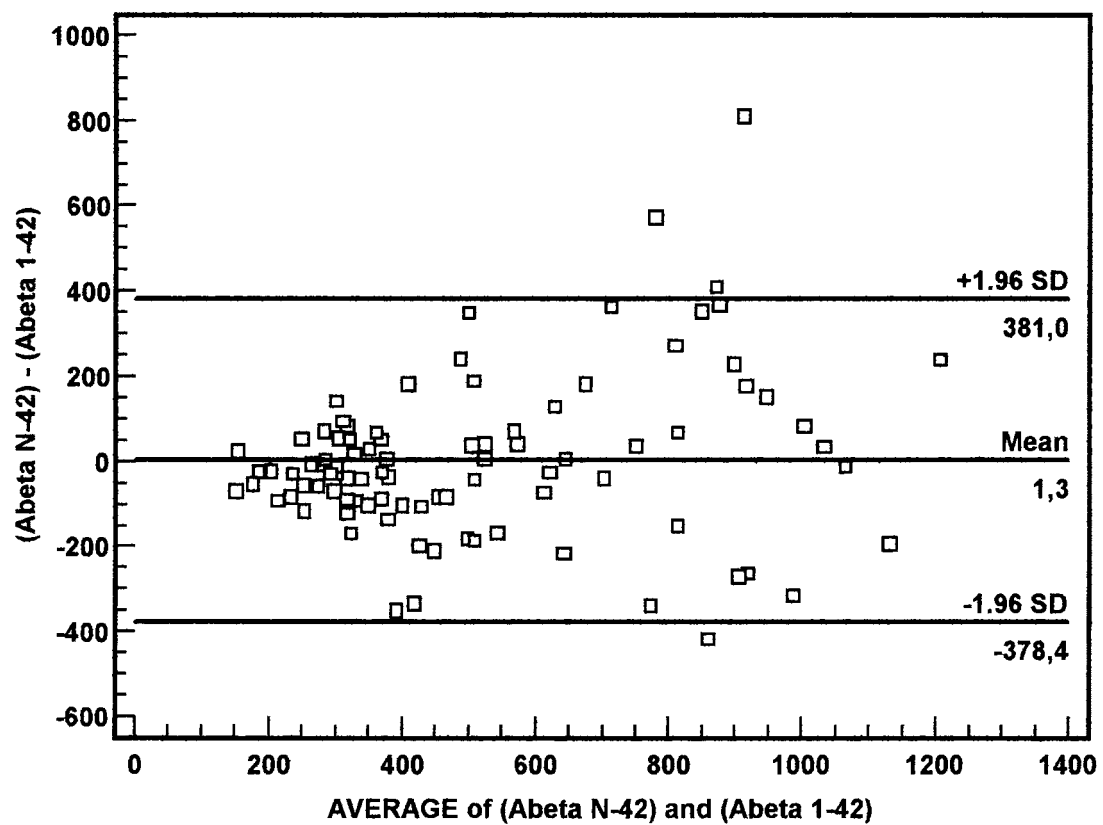


Figure 2



## Chapter 8.1

### Spike recovery of CSF A $\beta$ 42 concentration

Table 1

|   | Sample1<br>(pg/mL) | Sample 2<br>(pg/mL) | Sample 3<br>(pg/mL) | Sample 4<br>(pg/mL) |
|---|--------------------|---------------------|---------------------|---------------------|
| Undiluted<br>(pg/mL)  | 438                | 520                 | 266                 | 319                 |
| Measured A $\beta$ 42<br>concentration<br>(calculated<br>concentration) | 981(1119)          | 917(1260)           | 896(1133)           | 875(1160)           |
| Recovery (%)  | 76.2               | 72.7                | 79.0                | 75.4                |

The spike recovery of CSF A $\beta$ 42 was evaluated in two different runs by spiking four CSF samples 1:1 with the highest standard of the INNOTEST<sup>TM</sup>  $\beta$ -amyloid (1-42) ELISA, i.e. 2000 pg/mL. Recoveries ranged from 75.4 to 79% with an overall mean recovery of 76%.



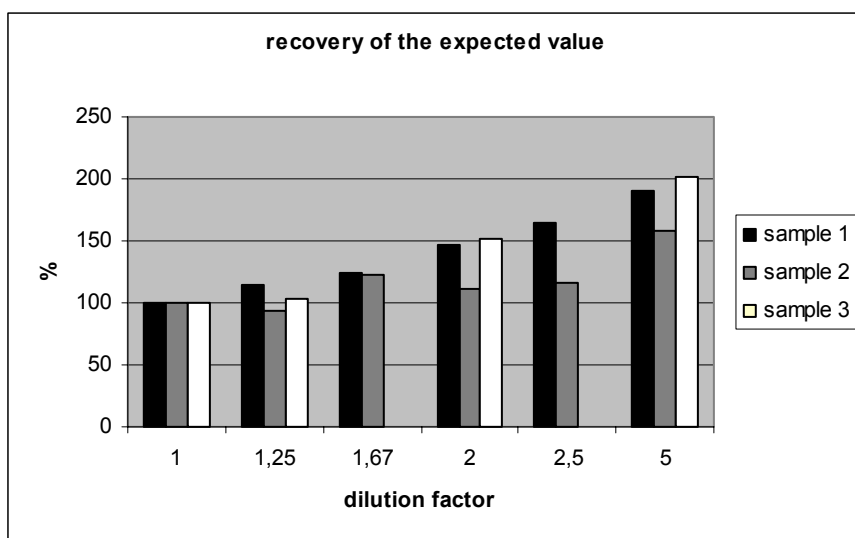
Recovery of expected value of CSF A $\beta$ 42 concentration

Table 2

| Sample +<br>sample<br>diluent | Sample 1<br>(pg/mL) | Recovery<br>of exp<br>value (%) | Sample 2<br>(pg/mL) | Recovery of<br>exp value<br>(%) | Sample 3<br>(pg/mL) | Recovery<br>of exp<br>value (%) |
|-------------------------------|---------------------|---------------------------------|---------------------|---------------------------------|---------------------|---------------------------------|
| 100 + 0                       | 1055                | 100                             | 806                 | 100                             | 1124                | 100                             |
| 80 + 20                       | 966                 | 114                             | 605                 | 94                              | 930                 | 103                             |
| 60 + 40                       | 802                 | 125                             | 593                 | 122                             | -----               | ----                            |
| 50 + 50                       | 778                 | 147                             | 446                 | 111                             | 851                 | 151                             |
| 40 + 60                       | 696                 | 165                             | 375                 | 116                             | -----               | -----                           |
| 20 + 80                       | 403                 | 191                             | 255                 | 158                             | 451                 | 201                             |

In three different runs three CSF samples were assayed at five serial dilutions with sample diluent. In Table 2 the percent recovery of expected value (or linearity) is listed. Recoveries ranged from 158 to 201% in the experiment with the highest dilution (1:5). See also Figure 1.

Figure 1 Recovery of the expected value of CSF A $\beta$ 42 concentration



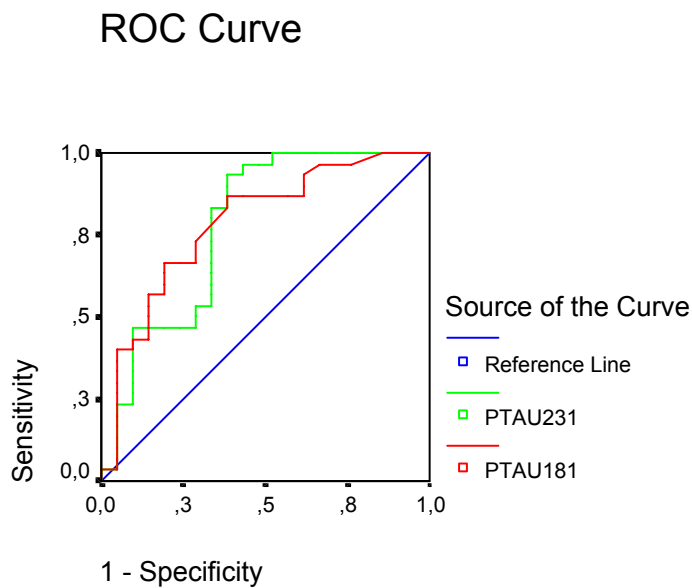
## ***Chapter 8.1***

Criteria for an ideal biomarker for AD<sup>38</sup>:

- Reflection of neuropathological changes in AD
- Gold standard: at autopsy neuropathological changes typical for AD
- Sensitivity  $\geq 85\%$
- Specificity  $\geq 75\%$
- Able to detect AD early on the course of disease
- Useful in monitoring disease progression and treatment effect
- Reliable and precise
- Non-invasive and simple procedure
- Not expensive

## Chapter 8.1

ROC curve CSF Ptau-231 and Ptau-181 in AD (N = 30) versus FTD (N =21)



Diagonal segments are produced by ties.

Ptau-181, cut off 55 pg/ml, sensitivity = 87%, specificity = 62%

Ptau-231, cut off 40 pg/ml, sensitivity = 87%, specificity = 62%

Areas under the curve (95% CI):

Ptau-181 = 0.79 (0.66-0.92)

Ptau-231 = 0.78 (0.64-0.92)

## Chapter 8.1

### MTA in EAD and LAD versus age-matched controls

Table 3

|                       | < 65 years old |           |        | ≥ 65 years old |          |       |
|-----------------------|----------------|-----------|--------|----------------|----------|-------|
|                       | AD             | Controls  | P      | AD             | Controls | P     |
| Average MTA (min-max) | 1 (0-2.5)      | 0 (0-0.5) | <0.001 | 1.5 (0-3.5)    | 0 (0-3)  | 0.001 |

MTA = medial temporal lobe atrophy; Average MTA:  $MTA\ R+L/2$ ; P = P-value comparing AD with controls (Mann Whitney U test).

## Chapter 8.2

### Reference values of the VUMC

AD:

$A\beta_{42} < 500 \text{ pg/mL}$

$\text{Tau} > 350 \text{ pg/mL}$

$\text{Ptau-181} > 60 \text{ pg/mL}$

Controls:

$A\beta_{42} \geq 500 \text{ pg/mL}$

$\text{Tau} \leq 350 \text{ pg/mL}$

$\text{Ptau-181} \leq 60 \text{ pg/mL}$

|                           | $A\beta_{42}$      | Tau                | Ptau              |
|---------------------------|--------------------|--------------------|-------------------|
| Alzheimer's disease       | Decreased          | Increased          | Increased         |
| Lewy Body Dementia        | Decreased          | Normal             | Normal            |
| Vascular Dementia         | Normal - decreased | Normal - increased | Normal            |
| Frontotemporal Dementia   | Normal - decreased | Normal – increased | Normal            |
| Creutzfeldt Jakob Disease | Normal - decreased | Strongly increased | Relatively normal |
| Non-demented elderly      | Normal             | Normal             | Normal            |



## SUMMARY

The diagnose Alzheimer's disease (AD) is based on clinical criteria, supported by neuropsychological tests, neuroimaging and extended follow up. A definite diagnose can only be obtained at autopsy. With the advent of disease modifying therapeutics, it is important to diagnose AD as early as possible, before extensive brain damage has occurred. In this early stage biochemical markers are needed for diagnosis as clinical symptoms are subtle and other diagnostic methods fairly normal. Up to now amyloid  $\beta$  42 ( $A\beta$ 42), total tau (tau) and phosphorylated tau (Ptau), as measured in cerebrospinal fluid (CSF), are considered to be the most promising biochemical markers for AD. The present thesis tells us how and when to use these markers for the early and differential diagnosis AD in clinical practice.

We conclude that the triplet of  $A\beta$ 42, tau and Ptau must be measured in CSF in order to differentiate AD from normal aging and other types of dementias. Particularly useful are these biomarkers in presenile dementias –starting before 65 years old- as the differential diagnosis here is wider and more complicated. At least two of the markers must be abnormal for the diagnosis AD, while all three markers negative can practically rule out the disease. CSF  $A\beta$ 42, tau and Ptau are potential markers to predict AD in the preclinical stage of disease. However, in this early stage the markers are not independent from other clinical parameters. Moreover, the combination of the three CSF markers and atrophy of the medial temporal lobe are of incremental value for the diagnosis AD. Hence, the three CSF biomarkers  $A\beta$ 42, tau and Ptau can only be used as diagnostic tool *in addition to* other diagnostic methods. Variability of CSF  $A\beta$ 42 concentrations is explained by pre-analytical and internal (ELISA) factors. Current (commercial) ELISAs are quite reliable as diagnostic method compared to the other methods available.





## SAMENVATTING

De diagnose ziekte van Alzheimer (AD) wordt gesteld op basis van klinische criteria, met ondersteuning van neuropsychologische tests, beeldvorming en langdurige follow-up. De definitieve diagnose kan alleen worden vastgesteld bij obductie. Van belang is om de diagnose in een zo vroeg mogelijk stadium te stellen zodat, voordat er in de hersenen schade is aangericht, kan worden gestart met therapie. Juist in dit preklinische stadium van de ziekte is er behoefte aan biochemische markers, aangezien de klinische symptomen subtiel zijn, en andere diagnostische methoden vaak normaal. Liquor cerebrospinalis (cerebrospinal fluid, CSF) wordt verondersteld een goede afspiegeling te geven van wat zich in de hersenen afspeelt. Tot nu toe is gebleken dat bepaling van A $\beta$ 42, totaal tau (tau) en Ptau in CSF erg sensitief is voor de diagnose AD. In dit proefschrift wordt uiteengezet hoe en wanneer deze drie biochemische markers het beste kunnen worden gebruikt voor de diagnose AD in een klinische setting.

Alledrie de markers zijn nodig om AD te kunnen onderscheiden van normale veroudering en verschillende soorten dementie. Voor de diagnose AD moeten twee van de drie markers positief zijn, terwijl als alledrie de markers negatief zijn de ziekte vrijwel is uitgesloten. De markers zijn met name van belang bij het onderscheid tussen verschillende vormen van preseniele dementie (ontstaan van klachten voor het 65<sup>ste</sup> jaar), waarbij de differentiële diagnose uitgebreid is, en overige diagnostische methoden niet eenduidig. CSF A $\beta$ 42, tau en Ptau zijn veelbelovend om te gebruiken bij het voorspellen van AD in een preklinisch stadium. Echter, ze zijn niet onafhankelijk van andere klinische variabelen, en dragen supplementair bij aan de diagnose AD in combinatie met een MRI scan. Geadviseerd wordt om de markers alleen te bepalen naast andere diagnostische methoden. De variabiliteit van de A $\beta$ 42 concentratie in CSF wordt veroorzaakt door preanalytische en interne (ELISA) factoren. De gangbare (commerciele) ELISA's zijn net zo goed als diagnostische methode vergeleken met andere analysemethoden.



## DANKWOORD

Geen proefschrift zonder dankwoord. Wetenschap bedrijven binnen de geneeskunde is niet eenzaam, aan één artikel werken tenminste 6 mensen mee. In feite nog meer, als je rekent vanaf de patiënt naar de lumbaalpunctie via het lab, de analyse van data, de vergaderingen, koffiepauzes, praatjes, congressen, etc. Pas in de laatste fase sta je er min of meer alleen voor, met als moment suprême: de verdediging van het ‘boekje’. Daarom wilde ik graag iedereen bedanken die op een of andere manier betrokken is geweest bij het stand komen van dit proefschrift en niet in de laatste plaats de patiënten zelf en hun familie. In het bijzonder gaat mijn aandacht naar:

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## **CURRICULUM VITAE**

Niki Schoonenboom (03-03-1972) is in Wassenaar opgegroeid en voltooide daar het gymnasium  $\beta$  aan het Rijnlands Lyceum in 1990. Ze is in hetzelfde jaar geneeskunde gaan studeren aan de Universiteit van Amsterdam. Na de studie werd de interesse voor de oudere mens gewekt als AGNIO ouderenpsychiatrie bij de Robert Fleury Stichting in Leidschendam, en later verder als AGNIO neurologie in het Slotervaartziekenhuis in Amsterdam (1999). In 2000 begon ze met promotieonderzoek bij prof.dr. P. Scheltens, Alzheimer Centrum, VUmc (2000-2004). In 2002 kreeg ze de gelegenheid om 5 maanden deelonderzoek te doen in New York, bij het 'Center for Brain Health' van NYU, en het 'Institute for Basic Research in Developmental Disabilities', Staten Island, onder leiding van respectievelijk prof.dr. MJ De Leon en dr. PD Mehta. Eind 2004 kon ze beginnen als AGIO neurologie in het VUmc, waarmee ze hopelijk in 2010 klaar zal zijn.



## **CURRICULUM VITAE**

Niki Schoonenboom (03-03-1972) grew up in Wassenaar, and graduated in 1990 from the ‘Rijnlands Lyceum’ (Gymnasium  $\beta$ ). The same year she moved to Amsterdam (University of Amsterdam) to study Medicine. After obtaining her medical degree, she started her career as physician at Geriatric Psychiatry (Robert Fleury Stichting, Leidschendam) and Neurology (Slotervaartziekenhuis, 1999). From 2000-2004 she worked on her PhD thesis under the supervision of prof.dr. P Scheltens at the Alzheimer Center of the VUmc. In 2003 she got the opportunity to do a research project at the Center for Brain Health, NYU, New York, and the Institute for Basic Research in Developmental Disabilities, Staten Island, US, under the supervision of prof.dr. MJ De Leon and dr. PD Mehta respectively. Since 2004 she works as a resident in Neurology at the VUmc, which she hopefully will conclude in 2010.

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